

University of Denver

Digital Commons @ DU

Electronic Theses and Dissertations

Graduate Studies

8-1-2014

Mitochondrial Involvement in Neuronal Cell Death

Josie J. Gray

University of Denver

Follow this and additional works at: <https://digitalcommons.du.edu/etd>



Part of the [Biochemistry, Biophysics, and Structural Biology Commons](#)

Recommended Citation

Gray, Josie J., "Mitochondrial Involvement in Neuronal Cell Death" (2014). *Electronic Theses and Dissertations*. 244.

<https://digitalcommons.du.edu/etd/244>

This Dissertation is brought to you for free and open access by the Graduate Studies at Digital Commons @ DU. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Digital Commons @ DU. For more information, please contact jennifer.cox@du.edu, dig-commons@du.edu.

MITOCHONDRIAL INVOLVEMENT IN NEURONAL CELL DEATH

A Dissertation

Presented to

The Faculty of Natural Sciences and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

Josie J. Gray

August 2014

Advisor: Daniel A. Linseman

©Copyright by Josie J. Gray 2014

All Rights Reserved

Author: Josie J. Gray

Title: MITOCHONDRIAL INVOLVEMENT IN NEURONAL CELL DEATH

Advisor: Daniel A. Linseman

Degree Date: August 2014

Neuronal cell death via apoptosis or necrosis underlies several devastating neurodegenerative diseases associated with aging. Mitochondrial dysfunction resulting from oxidative or nitrosative stress often acts as an initiating stimulus for intrinsic apoptosis or necrosis. These events frequently occur in conjunction with imbalances in the mitochondrial fission and fusion equilibrium, although the cause and effect relationships remain elusive. In this thesis, I demonstrate in primary rat cerebellar granule neurons (CGNs) that oxidative or nitrosative stress induces an N-terminal cleavage of optic atrophy-1 (OPA1), a dynamin-like GTPase that regulates mitochondrial fusion and maintenance of cristae architecture. This cleavage event is indistinguishable from the N-terminal cleavage of OPA1 observed in CGNs undergoing caspase-mediated apoptosis (Loucks et al., 2009) and results in removal of a key lysine residue (K301) within the GTPase domain. OPA1 cleavage in CGNs occurs coincident with extensive mitochondrial fragmentation, disruption of the microtubule network, and cell death. In contrast to OPA1 cleavage induced in CGNs by removing depolarizing extracellular potassium (5K apoptotic conditions), oxidative or nitrosative stress-induced OPA1 cleavage caused by complex I inhibition or nitric oxide, respectively, is caspase-*independent*. N-terminal cleavage of OPA1 is also observed *in vivo* in aged rat and mouse midbrain and hippocampal tissues. We conclude that N-terminal cleavage and subsequent inactivation of OPA1 may be a contributing factor in the neuronal cell death processes underlying neurodegenerative diseases, particularly those associated with aging.

Furthermore, these data suggest that OPA1 cleavage is a likely convergence point for mitochondrial dysfunction and imbalances in mitochondrial fission and fusion induced by oxidative or nitrosative stress.

Protein aggregation is a common pathogenic mechanism in diverse neurodegenerative disorders. TAR DNA-binding protein-43 (TDP-43) cytoplasmic inclusions are pathological hallmarks of several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Furthermore, TDP-43 mutations have been identified in both familial and sporadic forms of ALS. Despite evidence linking dysfunction of TDP-43 to neuronal cell death, it is currently unknown whether TDP-43-induced toxicity occurs via a gain of function or loss of function mechanism. In the present study, I initially examined the effects of either overexpression or knockdown of TDP-43 on neuronal survival *in vitro*. Overt neuronal cell death was not induced by either experimental paradigm. However, it was noted that both endogenous and overexpressed TDP-43 partially localized to mitochondria within diverse neuronal and astrocytic cell types. Furthermore, a significant decrease in the function of mitochondrial ETC Complex I occurred following TDP-43 knockdown in NSC34 motor neuron/neuroblastoma hybrid cells. These data suggest a novel link between TDP-43 localization and function and mitochondrial physiology.

Despite the preponderance of evidence linking TDP-43 dysfunction to neuronal cell death, current investigations indicate that the mechanism by which this protein exerts its toxicity is very complex. Furthermore, little insight has been given into the regulation of TDP-43 nuclear export and cytoplasmic localization. Previously, we described that TDP-43 consistently localizes to the mitochondria in diverse neuronal cell types. In

addition, siRNA-mediated TDP-43 depletion was associated with a deficit in mitochondrial ETC complex I. In the present study, I report that TDP-43 nuclear export is enhanced by reductions in mitochondrial glutathione (GSH) levels. Accordingly, redistribution of TDP-43 from the nucleus to the cytoplasm is induced by ethacrynic acid (EA) depletion of cellular glutathione levels, and this effect is inhibited by overexpression of the mitochondrial GSH transporter, the 2-oxoglutarate carrier (OGC), which specifically increases the mitochondrial GSH pool. Furthermore, inhibition of c-Jun N-terminal kinase (JNK) but not p38 MAP kinase effectively ameliorates EA induced TDP-43 nuclear export. These data suggest novel regulation of TDP-43 cellular distribution and highlights a relationship between mitochondrial physiology, TDP-43 dysfunction, and neuronal cell death.

Acknowledgements

I would like to thank my thesis advisor, Daniel Linseman, for his support over the years and the opportunity to learn through his mentorship. I would also like express gratitude to my graduate thesis committee, who have continually motivated me to “think outside the box” – Scott Barbee, Todd Blankenship, Nancy Lorenzon, Tom Quinn, and my outside chair, Gareth Eaton. In addition, I would like to acknowledge members of the Linseman lab - Trisha Stankiewicz, Aimee Winter, Faten Ibrahim, and Heather Wilkins. I would like to thank those faculty and instructors that I have taught with over the years – Kristin Andrud, Angela Hebel, Julie Morris, and Nancy Sasaki. Finally, I would like to extend my gratitude to my original mentor in science, who convinced me some years ago to go back to graduate school, Loris McGavran.

To my wonderful family, especially my parents, my brother, and niece. Your support of me over the years has been the foundation for the conviction and tenacity that I pursue my goals with. I am forever grateful for the unconditional love you have always given me. I would like to extend my warmest gratitude to Heather Hodgin, for always being there for me (for many years), and being someone I can always look to and look up to. I am forever grateful for you in my life. To Reed Hodgin, for his mentorship and support. In addition, I would like to thank the rest of the Hodgin Family for their love throughout the years. To Julianna West and Bonnie Albrecht – no matter how far or how long, you have both always been there for me and I love you dearly. To Jen Ikle and Crystal Woods, for always giving me guidance and having open arms and open ears for me. Your friendship has been such an anchor for me during my journey on this somewhat unpredictable path.

Table of Contents

Chapter One: Introduction	1
1.1 Mitochondrial dynamics	1
1.1.1 Mitochondrial dynamics and fission/fusion machinery	1
1.1.2 OPA1	11
1.2 Mitochondrial oxidative stress and dysfunction in neurodegeneration	19
1.2.1 Mitochondrial electron transport chain and redox biology	20
1.2.2 Oxidative stress and mitochondrial dysfunction in the etiology of neurodegeneration	23
1.2.3 Glutathione and neurodegeneration	28
1.3 Trans-activating Response DNA binding Protein/ TARDBP/ TDP-43	35
1.3.1 TDP-43 Structure and Function	36
1.3.2 TDP-43 aggregation and ubiquitination in ALS	40
1.3.3 TDP-43 Mutations in ALS	43
1.3.4 Loss of Function verses Gain of Function and TDP-43 Toxicity	45
Chapter Two: N-terminal cleavage of the mitochondrial fusion GTPase OPA1 occurs via a caspase- <i>independent</i> mechanism in cerebellar granule neurons exposed to oxidative or nitrosative stress	49
2.1 Abstract	49
2.2 Introduction	50
2.3 Materials and Methods	54
2.3.1 Materials	54
2.3.2 Cell Culture	55
2.3.3 Brain tissue preparation	55
2.3.4 Cell lysis and immunoblotting	56
2.3.5 Immunofluorescence/mitochondrial staining	56
2.4 Results	57
2.4.1 5K-induced CGN apoptosis triggers caspase-dependent N-terminal OPA1 cleavage	57
2.4.2 Mitochondrial fragmentation occurs in concert with N-terminal cleavage of OPA1.	60
2.4.3 Complex I inhibition with MPP ⁺ induces caspase-independent N-terminal cleavage of OPA1 that is otherwise indistinguishable from that observed during 5K-induced apoptosis	63
2.4.4 MPP ⁺ induces striking mitochondrial fragmentation and complete disruption of the microtubule network in CGNs	67
2.4.5 Rotenone-induced oxidative stress causes caspase-independent OPA1 cleavage, mitochondrial fragmentation, and microtubule disruption	69
2.4.6 Reactive nitrogen species (RNS) induces caspase-independent N-terminal cleavage of OPA1, mitochondrial fragmentation, and microtubule disassembly	72
2.4.7 OPA1 cleavage induced by rotenone is resistant to inhibitors of JNK and p38 MAP kinases	75

2.4.8 N-terminal cleavage of OPA1 occurs in aged rat and mouse brain tissues	77
2.5 Discussion	80
Chapter Three: TAR-DNA binding protein-43 (TDP-43) localization to mitochondria suggests a novel role in modulating mitochondrial physiology.....	
3.1 Abstract	90
3.2 Introduction.....	91
3.3 Materials and Methods.....	93
3.3.1 Materials	93
3.3.2 Cell Culture.....	94
3.3.3 Transfection procedure	94
3.3.4 Cell lysis and immunoblotting	95
3.3.5 Immunofluorescence.....	95
3.3.6 Cell Fractionation.....	96
3.3.7 MTT viability assay	96
3.3.8 SMARTPOOL [®] siRNA mediated knockdown of TDP-43.....	97
3.3.9 Oroboros	97
3.4 Results.....	98
3.4.1 Overexpression of WT or A315T mutant TDP-43 is not overtly toxic to NSC-34 motor neuronal cells.	98
3.4.2 Depletion of cellular TDP-43 is not toxic to NSC-43 cells	101
3.4.3 Endogenous and GFP-tagged TDP-43 partially localize to mitochondria	103
3.4.4 Knockdown of TDP-43 induces mitochondrial Complex I deficiency	105
3.5 Discussion	109
Chapter Four: TDP-43 nuclear export is partially mediated by mitochondrial glutathione levels and c-Jun N –Terminal Kinase	
4.1 Abstract	113
4.2 Introduction.....	114
4.3 Materials and Methods.....	115
4.3.1 Materials	115
4.3.2 Cell Culture.....	116
4.3.4 Cell lysis and immunoblotting.....	116
4.3.5 Immunofluorescence.....	117
4.3.6 Cell Fractionation.....	117
4.3.7 Glutathione Assay	118
4.4 Results.....	118
4.4.1 Depletion of cellular GSH levels induces nuclear export of TDP-43	118
4.4.2 Stable expression of the mitochondrial OGC carrier specifically increases mitochondrial GSH levels.	121
4.4.3 Stable overexpression of the mitochondrial OGC carrier prevents nuclear export of TDP-43.	123

4.4.4 Nuclear export of TDP-43 is ameliorated by inhibition of JNK but not p38 MAP kinase.....	125
4.5 Discussion	127
Chapter Five: Conclusion	129
5.1 Summary of major findings	129
5.2 Discussion	131
References	138
Appendix A: Abbreviations	159
Appendix B: Publications	161

List of Figures

1.1.1 - Schematic of vertebrate mitochondrial fission and fusion components	10
1.1.2 - Structural domains of OPA1 and exon splice sites	14
1.1.3 - OPA1 oligomerization and sequestration of cytochrome <i>c</i>	17
1.2.4 – Synthesis of glutathione	30
1.3.1 - TDP-43 Protein Domains	39
2.4.1 - Removal of depolarizing extracellular potassium (5K) induces N-terminal cleavage of OPA1 that is caspase-dependent.....	59
2.4.2 - CGNs display significant mitochondrial fragmentation but minimal microtubule disruption following 5Ktreatment.....	61
2.4.3 - OPA1 cleavage induced by the complex I inhibitor, MPP ⁺ , is indistinguishable from 5K-induced OPA1 cleavage but is caspase- <i>independent</i>	65
2.4.4 - MPP ⁺ induces striking mitochondrial fragmentation and severe microtubule disruption in CGNs	68
2.4.5 - OPA1 cleavage induced by the complex I inhibitor rotenone is caspase-independent and occurs concurrently with mitochondrial and β -tubulin fragmentation ..	70
2.4.6- OPA1 cleavage induced by the nitric oxide donor, SNP, is caspase-independent and occurs concurrently with mitochondrial fragmentation and microtubule disassembly	73
2.4.7 - OPA1 cleavage induced by 5K treatment or rotenone demonstrate differential resistance to inhibition of JNK and p38 MAP kinase.....	76
2.4.8 - OPA1 cleavage is observed in aged midbrain and hippocampal tissues	78
2.4.9 - Schematic of proposed OPA1 cleavage mechanism	89
3.4.1 - Overexpression of WT or A315T mutant TDP-43 is not overtly toxic to NSC-34 motor neuronal cells.....	100
3.4.2 - Depletion of cellular TDP-43 expression levels is not toxic to NSC-43 cells	102
3.4.3 - Endogenous and GFP-tagged TDP-43 partially localize to mitochondria	104
3.4.4 - Knockdown of TDP-43 induces mitochondrial Complex I deficiency	107
4.4.1 - Depletion of Cellular GSH Levels Induces Nuclear Export of TDP-43	120

4.4.2 - Stable expression of the mitochondrial 2-oxoglutarate effectively increases mitochondrial GSH levels.....	122
4.4.3 - Stable overexpression of the mitochondrial 2-oxoglutarate carrier prevents nuclear export of TDP-43	124
4.4.4 - Nuclear export of TDP-43 is ameliorated by inhibition of JNK but not p38 MAP kinase	126

Chapter One: Introduction

1.1 Mitochondrial dynamics

1.1.1 Mitochondrial dynamics and fission/fusion machinery

Mitochondria are necessary organelles, whose existence and function are indispensable for cell survival. Although most mitochondrial proteins are encoded in the nucleus, there is a subset of proteins that are encoded by the mitochondrial genome. The mitochondria have fundamental roles within the cell, with their primary purpose being to produce the cell's energy in the form of ATP. This function is partially allowed for by the double-membrane bound structure of the mitochondria. The inner membrane, where the energy producing respiratory chain complexes are located, is arranged in folds that form the mitochondrial cristae. Regulation of mitochondrial morphology is fundamental in its ability to function, and the importance of this organelle is perhaps highlighted by the intricate nature in which mitochondria form and function are maintained within the cell.

Mitochondria are dynamic organelles, whose ever changing nature is an interesting contrast to the somewhat static morphology of many other cellular organelles. They exist not as individual entities but as a network which ranges from smaller, individual units to a more tubular, interconnected system. The fluctuation between these two states is termed “mitochondrial dynamics”, and occurs through the fission and fusion of inner and outer membranes of the individual organelles. The overall health of the

mitochondria relies on a critical balance of these two opposing forces. If mitochondrial fusion components are impaired, the mitochondria adopt a fragmented morphology as a result of unopposed fission processes. Likewise, if mitochondrial fission components are defective, the mitochondria become excessively elongated and dysfunctional due to unopposed fusion (Bleazard et al., 1999; Sesaki et al., 1999; Chen et al., 2005). The state of the fission-fusion spectrum varies between cell types and even within the same cell type, depending on the biological and energy needs. For instance, mitochondrial morphology changes throughout the cell cycle, containing a longer, interconnected morphology during G1/S phases and adopting a more fragmented morphology during mitosis (Mitra et al., 2009). Based on a significant number of studies that substantiate the importance of correctly balanced mitochondrial fission and fusion processes, it is widely accepted that the function of mitochondrial dynamics goes far beyond simple, morphologic regulation of the organelle. Although the method by which these organelles achieve such a dynamic existence is very complex, much has been discovered about the proteins which carry out the processes of fission and fusion.

Mitochondrial Fission

One of the central purposes of mitochondrial fission is to multiply mitochondrial numbers in order to repopulate growing and dividing cells (Youle & van der Bliek, 2012). Another, perhaps even more important function of fission properties, is the execution of mitochondrial quality control. As certain components of the mitochondria become damaged, mitochondria are able to segregate the dysfunctional portions to a “daughter” mitochondrion, which is targeted for destruction through mitophagy, leaving the healthy components in the remaining “daughter” mitochondrion (Twig et al., 2008).

Under more extreme, irreparable circumstances, mitochondrial fission is tightly coupled to complete cellular destruction through the apoptotic cascade. Accordingly, mitochondrial outer membrane permeabilization by Bax is shown to occur concomitantly with increased mitochondrial fission. Furthermore, the mitochondrial fission protein DRP1 is shown to co-localize with Bax foci, which precede the release of cytochrome *c* and its initiation of the apoptotic cascade (Suen et al., 2008). These tightly controlled processes underscore the importance the mitochondrial fission machinery in cellular function, as well as the complex nature in which fission participates in mitochondrial quality control.

Mitochondrial fission is carried out by proteins in the dynamin family of GTPases. DRP1 (dynamin-related protein 1) is a central participant in fission events. It assembles on the outer mitochondrial membrane where it uses its dynamin-GTPase function to constrict and pinch off the organelle into two separate entities (Bleazard, et al. 1999; Labrousse, et al. 1999; Sesaki and Jensen, 1999; Smirnova et al., 1999). Like dynamin, DRP1 contains a GTPase domain, a middle domain, and a GTPase effector domain (GED) (Fig. 1.1.1A). Unlike dynamin, however, DRP1 is lacking a plextrin homology domain (PH), indicating a need for additional factors at the mitochondria to recruit DRP1 to the outer mitochondrial membrane. Indeed, DRP1 is recruited to the outer mitochondrial membrane by a second class of fission proteins such as FIS1, MFF, miD49, and MiD51 (reviewed in Chan, 2012) (Fig. 1.1.1b).

The DRP1 mitochondrial recruitment machinery is well-defined in yeast systems. FIS1 (fission 1) contains a single transmembrane domain which anchors it to the outer mitochondrial membrane, while the majority of it remains cytosolically located (Fig.

1.1.1A). In *Saccharomyces cerevisiae*, FIS1 is clearly required for recruitment of yeast DRP1 ortholog DNM1 to the outer mitochondrial membrane (Mozdy, et al., 2000). Additionally, Mdv1 and Caf4 act as scaffolding proteins to bridge the interaction between FIS1 and Dnm1 (Tieu et al., 2000; Griffin et al., 2005). However straightforward the mechanism is in yeast, it becomes more perplexing in mammalian systems, as there are no mammalian orthologs to Mdv1 and Caf4 (reviewed in Chan, 2012). The role of mammalian FIS1 in recruitment of DRP1 to the mitochondria also appears to be more complex in mammalian cells. As predicted, overexpression of FIS1 in COS-7, Clone 9, and HeLa cells results in fragmented mitochondria, indicative of increased recruitment of DRP1 (Yoon et al., 2003; Stojanovski et al., 2004; Yu et al., 2005). These data are in accord with data demonstrating that inhibition of FIS1 in mammalian cells results in elongated mitochondria in several mammalian cell lines, seemingly due to a defect in DRP1 outer-membrane localization (Yoon et al., 2003; Lee et al., 2004; Stojanovski et al., 2004; Koch et al., 2005; Gandre-Babbe and van der Bliek 2008). Despite the evidence for a role of FIS1 in mitochondrial fission, knockdown of FIS1 does not alter the localization of DRP1 to the mitochondria in HeLa cells (Lee et al., 2004). These contrasting data are suggestive of complex mechanisms involved in recruitment of DRP1 to the mitochondria in mammalian cells. Indeed, other proteins, including MFF and MiD49/51, are shown to be involved in DRP1 mitochondrial recruitment (reviewed in Chan et al., 2012).

Like FIS 1, MFF (mitochondrial fission factor) is a protein anchored to the outer mitochondrial membrane (Fig 1.1.1A). Accordingly, inhibition of MFF results in a more elongated mitochondrial morphology (Gandre-Babbe and van der Bliek, 2008).

However, unlike knockdown of FIS1, inhibition of MFF does result in decreased mitochondrial recruitment of DRP1, indicating that it has a more direct function in this process (Otera et al., 2010). Furthermore, a direct physical association between DRP1 and MFF has been established (Lee et al., 2004, Otera et al., 2010). These data emphasize an important and direct role of MFF in DRP1 recruitment to the mitochondria.

MiD49/51 (MIEF1) (mitochondrial dynamics protein/mitochondrial elongation factor 1) also appear to function in DRP1 recruitment to the mitochondria, although the exact mechanism by which they act is somewhat unclear (Fig 1.1.1A-B). One study demonstrates that although inhibition of MiD 49 and 51 individually does not affect mitochondrial morphology, knockdown of both MiD49 and 51 simultaneously results in elongated mitochondria. This happens concomitantly with a defect in CCCP induced (and DRP1 dependent) mitochondrial fragmentation, suggesting the two may act in concert in DRP1 function at the mitochondria (Palmer et al., 2011). However, another study demonstrates that knockdown of MIEF1 (MiD 51) alone causes fragmented mitochondria, indicative of a fission defect (Zhao et al., 2011). Both studies reveal that, although overexpression of MiD49/51(MIEF1) does result in increased DRP1 recruitment to the mitochondria, it also causes the mitochondrial network to become elongated, indicating that DRP1 may not be active or able to function in its fission role. This is further supported by data indicating that fission morphology (and DRP1 GTPase activity) is restored upon knockdown of MiD51, suggesting that MiD51 (MIEF1) has a pro-fusion function (Zhao et al., 2011). The picture is further convoluted in that MiD51 (MIEF1) is demonstrated to associate with the fission protein hFIS1, independent of its interaction with DRP1. Interestingly, the MIEF1 fusion-phenotype is inhibited by

overexpression of hFIS1, suggesting that hFIS1 serves to inhibit the anti-fission/pro-fusion role of MIEF1. Taken together, it can be surmised that although MiD49/51 (MIEF1) play a role in recruitment of DRP1 to the mitochondria, they may actually inhibit the fission activity of DRP1. This inhibitory role of MiD51 (MIEF1) is balanced by proper expression levels of hFIS1, which may allow for DRP1 to function once recruited. Again, the sensitivity of the fission-fusion equilibrium is highlighted by the complexity of the interactions between the various fission components.

Mitochondrial Fusion

Mitochondrial fusion is somewhat controlled by the bioenergetic needs of the cell. It occurs during metabolically active conditions as a means to perform oxidative phosphorylation at maximum efficiency (Youle and van der Bliek, 2012). However, like fission, mitochondrial fusion is a key component of the mitochondrial quality control system. Mitochondria function on a mixture of nuclear encoded proteins and proteins encoded by the mitochondrial genome (mtDNA). Not only are mutations in the mitochondrial genome inherited heterogeneously, but spontaneous mutations within a single mitochondrion can occur independently of other mitochondria. In these situations, fusion and subsequent content mixing of mitochondria can serve to dilute out damaged mitochondrial components through complementation of mutated mtDNA with that of wild type mtDNA (Chan, 2012). Additionally, cellular and mitochondrial function is dependent on the coordination of proper ratios between different mitochondrial and nuclear components within each mitochondrion in a given cell. This would not be possible without the matrix and membrane content mixing that occurs during

mitochondrial fusion (Chan, 2012). Indeed, mitochondrial fusion allows for a more homogenous and cohesive mitochondrial population within a given cell type.

Fusion of the inner and outer mitochondrial membranes occurs during two distinct processes (Song et al. 2009). The resulting fused mitochondria consist of a mixture of the two membranes and matrix components. Like mitochondrial fission machinery, mitochondrial fusion machinery is comprised of members of the dynamin family of GTPases. MFN1 and 2 (Mitofusin) are dynamin GTPases responsible for fusion of the outer mitochondrial membrane. These proteins are tethered to the mitochondria through a single transmembrane domain, with cytosolically located heptad repeat regions and GTPase domain (Campello et al., 2012; Chan, 2012) (Fig 1.1.1A). In mouse MFN 1/2 knockout studies, mitochondria demonstrate a loss of fusion capabilities, resulting in mitochondria with a fragmented morphology (Chen et al., 2003; Koshiba et al., 2004; Chen et al., 2005;). It is apparent that fusion of the outer mitochondrial membrane is a result of interaction between Mitofusins on adjacent mitochondria, as mitochondria expressing wild type Mitofusins will not fuse with Mitofusin deficient mitochondria (Koshiba et al., 2004; Meeusen et al., 2004). Hetero- and homo-oligomeric complexes form through interactions of antiparallel coiled-coil regions in Mitofusins that are on adjacent mitochondria (Koshiba et al., 2004). Further conformational changes, likely the result of GTP hydrolysis, mediate the final fusion of the membrane (Fig 1.1.1B).

Optic atrophy-1 (OPA1) is a member of the dynamin GTPase family and is the major player in fusion of the inner mitochondrial membrane, as well as maintenance of cristae architecture. These events are orchestrated through oligomerization of differentially processed forms of OPA1 (named for its involvement in the autosomal

dominant disease Optic Atrophy 1) (Olichon et al., 2003; Cipolat et al., 2004) (Fig. 1.1.1B). It is the mammalian ortholog of yeast Mgm1, which was found to have a necessary role in fusion of the inner mitochondrial membrane in yeast (Wong et al., 2000; Sesaki et al., 2003). Depletion or total loss of OPA1 function causes a severe deficit of mitochondrial fusion, as indicated by mitochondria with a severely fragmented morphology, whereas overexpression of this protein results in elongated mitochondria in cells that otherwise have a more punctate mitochondrial morphology (Wong et al., 2000; Olichon et al., 2002, 2003; Cipolat et al., 2004; Griparic et al., 2004; Lee et al., 2004; Chen et al., 2005; Guillou et al., 2005; Griparic et al., 2007; Song et al., 2007; Song et al., 2009). Accordingly, mitochondrial fusion is completely prevented in cells containing null alleles of *OPA1*, further indicating that a shift in the expression of fission-fusion components results in a disruption of mitochondrial dynamics (Song et al., 2007; Song et al., 2009). Interestingly, unlike Mitofusin-mediated outer membrane fusion, it is not necessary for a functional OPA1 to be present on adjacent membranes for fusion of the inner mitochondrial membrane to occur (Song et al., 2009). This can be partially explained by OPA1 being able to directly bind the inner mitochondrial membrane and hydrolyze GTP, indicating that OPA1 function in fusion may be, at least somewhat, dictated through direct membrane interaction (Ban et al., 2010).

OPA1 is ubiquitously expressed in mammalian systems, although expression patterns vary between organisms and even between tissue types within the same organism. Increased expression levels are observed in metabolically active organs, such as, the retina, brain, liver, heart, and pancreas (Alexander et al., 2000; Delettre et al., 2000; Delettre et al., 2001; Akepati et al., 2008;). It can be surmised that, given the

higher energy demands of these tissues, OPA1 is preferentially expressed to promote the mitochondrial dynamics machinery to more of a fusion state, which is shown to be more supportive of energy production (Youle and van der Bliek, 2012).

Although the mitochondrial fusion machinery consists of a smaller number of proteins than that of the mitochondrial fission machinery, the regulation of fusion is just as complex and sensitive to the relative protein levels of the individual components. Moreover, regulation of OPA1 occurs not only through control of expression levels, but also through complicated post-translational processing that is even more intricately regulated (discussed below).

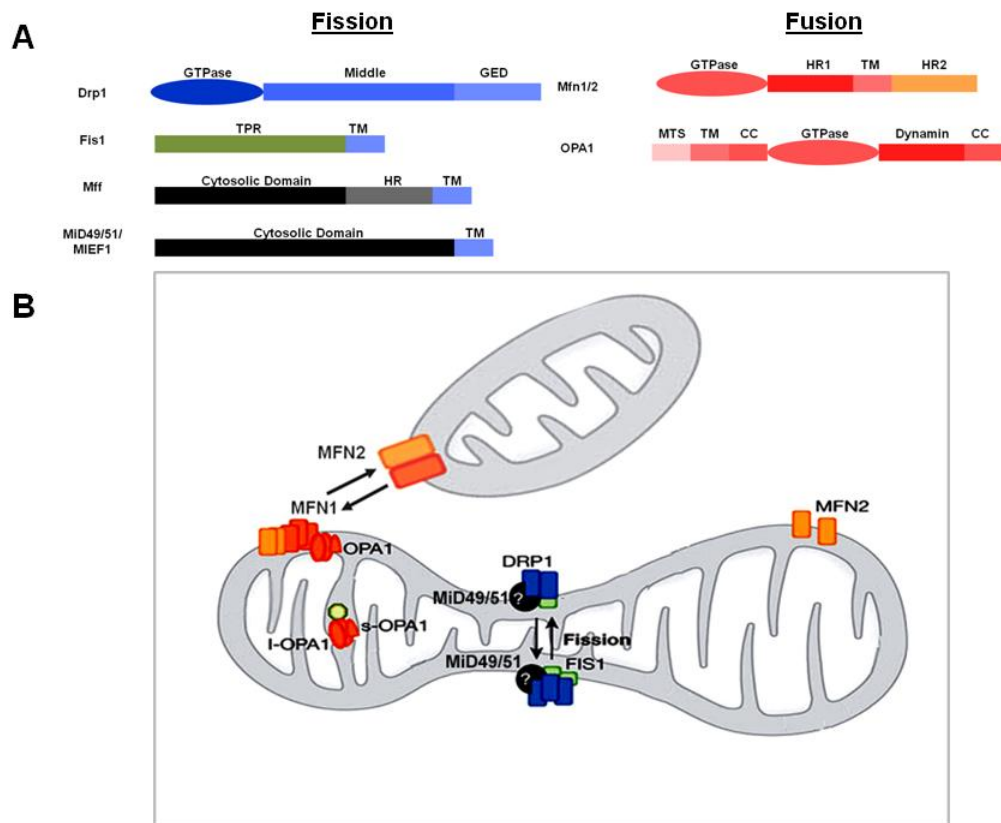


Fig. 1.1.1 – Schematic of vertebrate mitochondrial fission and fusion components. (A) Structural domains of Dynamin-related protein (DRP1) (blue), Fission 1 (FIS1) (green), Mitochondrial fission factor (MFF) (black/gray), mitochondrial dynamics protein/mitochondrial elongation factor 1 (MiD49/51/MIEF1) (black), Mitofusin 1/2 (MFN1/2) (red/orange), and Optic Atrophy 1 (OPA1) (red). (B) Schematic of physical interactions that facilitate fission and fusion processes. Mitochondrial fission occurs through putative cytoplasmic interactions between DRP1 (blue) and MiD49/51 (MIEF1) (black) and/or FIS1 (green), where DRP1 encircles and constricts through its GTPase function to form two new daughter mitochondria. Fusion of the outer membrane is facilitated through interactions of the cytosolically located heptad repeats (HR) between MFN1 and MFN2 (red/orange). Fusion of the inner membrane is performed by OPA1 (red), where the different isoforms also oligomerize to maintain cristae architecture (*adapted from Campello et al., 2010*). TPR=Tetratricopeptide Repeats, GED= GTPase Effector Domain, MTS= Mitochondrial Targeting Sequence, TM=Transmembrane, CC=Coiled Coil.

1.1.2 OPA1

Mutations in OPA1 were identified as causal in the neurodegenerative disease autosomal dominant optic atrophy 1 (ADOA-1), a progressive blindness disease in which patients carry one of several mutations in *OPA1*. As a result, they have progressive degeneration of the retinal ganglion cells, eventually causing atrophy of the optic nerve (Alexander et al., 2000; Delettre et al., 2000). Although the importance of OPA1 expression in retinal tissue is pathologically relevant, additional neuronal expression of OPA1 has been demonstrated in the motor cortex, the frontal brain, the spinal cord, and the cerebellar cortex (Delettre et al., 2001; Misaka et al., 2002; Aijaz et al., 2004). The importance of OPA1 expression in neurological function is evident, yet its expression in non-neural tissue indicates a widespread function.

OPA1 Protein domains and processing

OPA1 is a nuclear encoded member of the dynamin family of GTPases. As such, it contains a GTPase domain, a middle domain and a C-terminal coiled-coil GTPase effector domain (GED). Within the N-terminus of the protein, there is a basic mitochondrial import sequence (MIS) followed by three hydrophobic domains and another coiled-coil domain (reviewed in Landes et al., 2010) (Fig 1.1.2). Although the structure of OPA1 is not surprising, given that it belongs to the dynamin family of proteins, the post-translational processing and regulation of OPA1 elicits an intricate and complex story that remains somewhat perplexing.

OPA1 is assembled from thirty exons, of which exons 4, 4b, and 5b are alternatively spliced to yield eight mRNA variants (Delettre et al., 2001). Of note, exons 4b and 5b contain a hydrophobic transmembrane domain, which alludes to distinctive

roles of the alternatively spliced isoforms (Delettre et al., 2007; Duvezin-Caubet et al., 2007). Moreover, expression of the eight isoforms is highly tissue specific, indicating the necessity of differential processing of OPA1 based on the individual needs of the cell (Olichon et al., 2002; Akepati et al., 2008). Upon import into the mitochondria, the mitochondrial targeting sequence is cleaved by mitochondrial processing peptidases to produce a long (L) isoform (Olichon et al. 2002; Satoh et al., 2003; Ishihara et al., 2006). Within the mitochondria, OPA1 undergoes further cleavage at several positions (denoted S1 and S2) to yield soluble, short (S) isoforms. Of note, cleavage at S1 removes exon 4b and the preceding two transmembrane domains, whereas cleavage at S2 disrupts exon 5b and removes all three transmembrane domains (Fig 1.1.2). Although the exact driving force behind how OPA1 is processed post-transcriptionally is unclear, it does seem to be controlled, at least somewhat, at the alternative splicing level, as all isoforms containing exon 4b are consistently cleaved into the short isoform (Song et al., 2007). The long isoforms remain anchored to the inner membrane, whereas the shortest isoform is devoid of membrane anchoring domains. OPA1 is constitutively cleaved into the short isoform by the ATP-dependent metalloprotease YME1L1 at S2 (Griparic et al., 2007; Song et al., 2007). Additional cleavage events are mediated by the *m*-AAA proteases paraplegin and AFG3L1-2 at S1 during loss of mitochondrial membrane potential, and the presenilin-associated rhomboid-like (PARL) protease during apoptosis (Fig 1.1.2) (Herlan et al., 2003; Cipolat et al., 2006; Ishihara et al., 2006; Duvezin-Caubet et al., 2007; Griparic et al., 2007; Ishihara et al., 2007; Song et al., 2007; Guillery et al., 2008; Merkwirth et al., 2009). The overlap in actions of these proteases indicates a potential redundancy in their functions; this is highlighted by the observation that effective siRNA knock-down of any

one of these proteases does not abolish OPA1 processing (Griparic et al., 2007; Guillery et al., 2008). OPA1 functions in fusion and maintenance of cristae architecture relies on a critical balance of the various OPA1 spliceforms (and subsequent cleavage forms). Any disruption in these events causes a shift in this delicate equilibrium, which can result in mitochondrial fragmentation and disruption of the cristae architecture. Indeed, regulation of OPA1 processing is one of the tightly regulated components of the mitochondrial fission-fusion equilibrium.

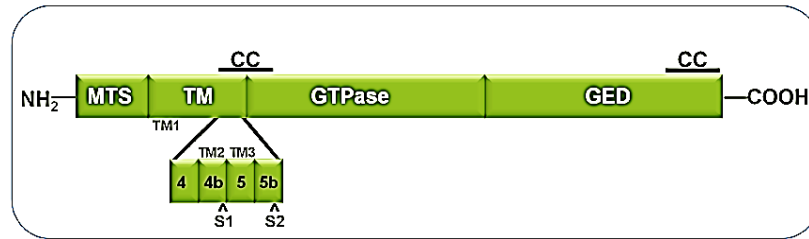


Fig. 1.1.2 - Structural domains of OPA1 and exon splice sites. OPA1 is comprised of an N-terminal mitochondrial targeting sequence (MTS) and a transmembrane domain (TM). The GTPase domain is followed by a C-terminal GTPase Effector Domain (GED). Coiled-coil (CC) domains flank the GTPase and GED domains. Exons 4, 4b, 5, and 5b are alternatively spliced to yield different mRNA products. Exons 4b and 5b contain additional transmembrane domains that can be spliced at sites S1 and S2, respectively, to yield additional OPA1 isoforms.

OPA1 in mitochondrial fusion

It is known that both long and short isoforms of OPA1 are necessary for proper mitochondrial fusion to take place in healthy cells. In fact, it is demonstrated that oligomerization of both isoforms is necessary for functional fusion to occur, indicating that they interact to facilitate fusion of the inner mitochondrial membrane (Meeusen et al., 2006; Song et al., 2007; Tondera et al., 2009). However, the precise mechanism by which OPA1 isoforms mediate mitochondrial fusion seems to be more complex. Evidence suggests that OPA1 oligomerization occurs through the coiled-coil domains, which is independent of the GTPase function (Akepati et al., 2008). Yet the GTPase domain and function are critical to the role of OPA1 in fusion, as mitochondrial fragmentation occurs in ADOA-1 patients containing a mutation in the GTPase domain (Spinazzi et al., 2008). These data suggest that although OPA1 oligomerization and GTPase mediated fusion capabilities are tightly coupled, they are each distinct and independent functions of the protein.

*OPA1 and cristae architecture and sequestration of cytochrome *c**

In addition to its function in mitochondrial fusion, OPA1 has a fundamental role in maintenance of mitochondrial cristae architecture. This is highlighted in studies which demonstrate that deficits in OPA1 result in disruption of cristae morphology (Olichon et al., 2003; Griparic et al., 2004; Frezza et al., 2006). It is understood that the different long and short cleavage products of OPA1 interact and oligomerizes to form complexes within the cristae to stabilize the cristae structure (Frezza et al., 2006). Tightly coupled to its role in maintenance of the cristae architecture is the role of OPA1 in sequestration of cytochrome *c* within the mitochondrial intermembrane space (Frezza et al., 2006;

Yamaguchi et al., 2008). This is a critical inhibitory process in the intrinsic apoptotic cascade, as release of cytochrome *c* is considered the initiation of the apoptotic cascade (Fig 1.1.3). As such, disruption in this function of OPA1 is shown to induce the intrinsic apoptotic cascade, in addition to causing mitochondrial fragmentation (Olichon et al., 2003; Cipolat et al., 2004; Lee et al., 2004; Olichon et al., 2007). Upon induction of apoptosis, OPA1 complexes are disrupted. Cytochrome *c* is subsequently released from the cristae into the cytosol, where it exerts its effects on the apoptotic cascade (Yamaguchi et al., 2008). The exact mode of OPA1 complexes in the retention of cytochrome *c* within the mitochondria is somewhat elusive. One possible method is that OPA1 functions as a diffusion barrier at the cristae junction, where it prevents cytochrome *c* release by physically retaining it within the cristae (Perkins et al., 2009). During apoptosis, OPA1 subunits become cleaved, causing disassembly of the complex and allowing for passage of cytochrome *c* into the cytosol. Additionally, cytochrome *c* is also proposed to be somewhat retained within the cristae through binding the mitochondrial inner membrane lipid cardiolipin, which is shown to interact with OPA1. This suggests that OPA1-mediated retention of cytochrome *c* may occur through indirect mechanisms as well (DeVay et al., 2009; Meglei et al., 2009).

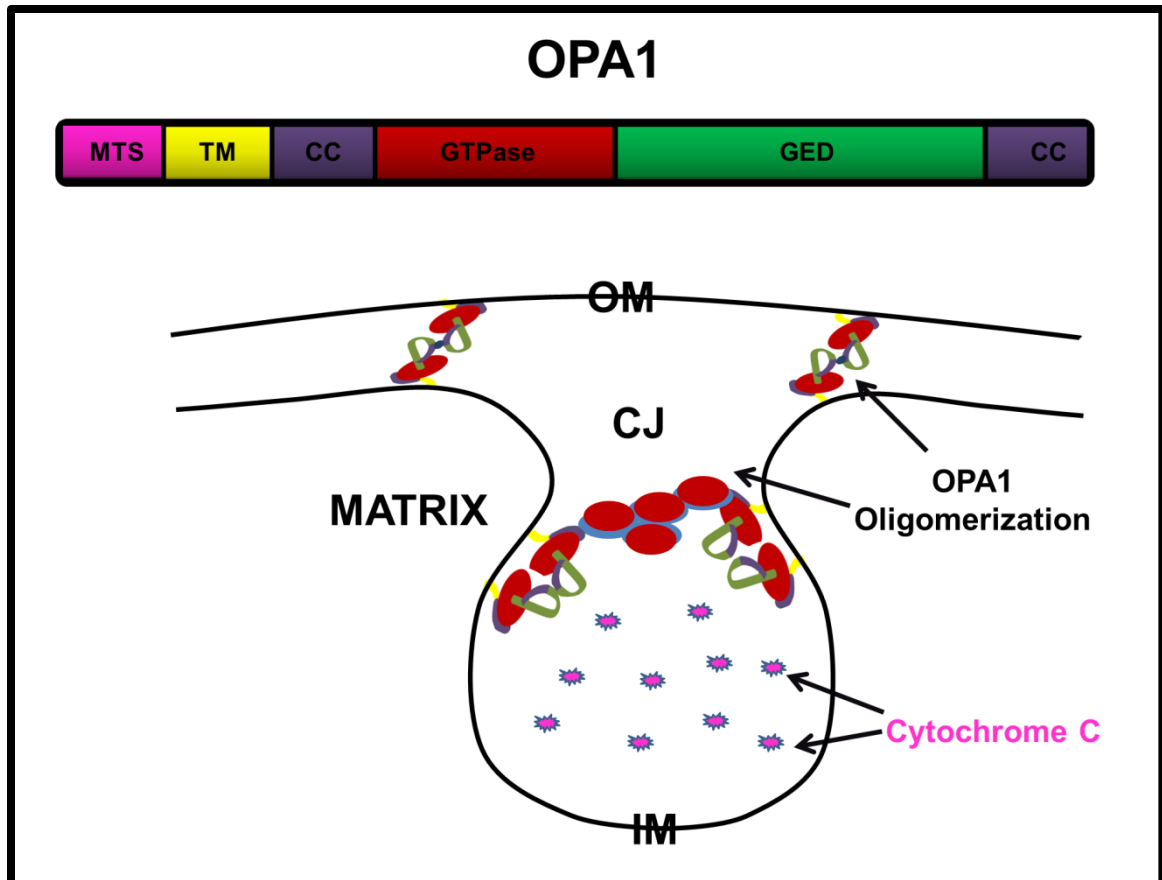


Fig. 1.1.3 - OPA1 oligomerization and sequestration of cytochrome *c*. OPA1 mitochondrial targeting sequence (MTS) is constitutively cleaved upon import into the mitochondria. It can anchor to the inner mitochondrial membrane (IM) through the transmembrane domain (TM) (yellow), or exist unanchored upon cleavage of the TM domain. OPA1 homo-oligomerization occurs through the coiled-coil (CC) (blue) domains (blue) and facilitates OPA1 fusion of the inner mitochondrial membrane (IM). OPA1 maintenance of cristae architecture is achieved through oligomerization of membrane anchored long isoforms and unanchored short isoforms. These associations allow an interaction that forms an effective blockade at the cristae junction (CJ) which sequesters cytochrome *c* (pink) within the mitochondrial cristae. OM= outer mitochondrial membrane; GED = GTPase effector domain (green).

The role of OPA1 in both fusion and retention of cytochrome *c* within the mitochondria is of critical importance, as it provides a mechanistic link between mitochondrial dynamics and apoptosis. The function of OPA1 is clearly very complex, and extends beyond its role in mitochondrial fusion. In fact, mitochondrial fusion and cristae architecture activities of OPA1 seem to be mechanistically distinct. This is highlighted in studies which demonstrate that expression of OPA1 mutants that are deficient in their fusion capabilities are still able to sequester cytochrome *c* and prevent apoptosis (Frezza et al., 2006). Furthermore, expression of disassembly resistant forms of OPA1 prevents the complete release of cytochrome *c* (Yamaguchi et al., 2008). The distinct functions of OPA1 can be isolated to specific OPA1 splice variants, as OPA1 isoforms including exon 4 are needed for fusion functions, whereas exons 4b and 5 seem to be more important for sequestration of cytochrome *c* (Olichon et al., 2007). The importance of both post-transcriptional and post-translational processing of this protein is evident, as proper splicing is needed in order for proper cleavage sites to be present. Although the mechanisms of OPA1 regulation are beginning to be unraveled, they seem to be just as complex as the function of this protein itself. The importance of OPA1 in mitochondrial fusion being independent from its role in cytochrome *c* sequestration is underscored by the complexity of how both functions occur, and that neither is dispensable for cell survival.

OPA1 involvement in mitochondrial defects

In vitro studies to delineate possible mechanisms by which OPA1 mutations cause disease have indicated that down regulation or loss-of-function of OPA1 results in mitochondrial fragmentation and disruption of the cristae architecture (Olichon et al., 2003; Griparic et al., 2004; Frezza et al., 2006). Specifically, adult mice carrying a heterozygous mutant allele of *OPA1* contain a more fragmented mitochondrial network (Davies et al., 2007). Moreover, fibroblasts from patients affected by ADOA-1 (resulting from a loss-of-function of one OPA1 allele) have a fragmented mitochondrial network compared to control individuals (Olichon et al., 2007; Chevrollier et al., 2008; Zanna et al., 2008). In addition to effects on mitochondrial morphology, OPA1 deficiency results in reduced mitochondrial respiratory capacity and increased susceptibility to apoptosis (Olichon et al., 2003; Chen et al., 2005; Frezza et al., 2006). These data further highlight the importance of OPA1 in mitochondrial function, and establish a mechanistic link between OPA1 deficiency and mitochondrial mediated cell death.

1.2 Mitochondrial oxidative stress and dysfunction in neurodegeneration

Neurodegenerative diseases include several illnesses, such as, Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease (AD), Parkinson's Diseases (PD), and Huntington's disease (HD). Despite the diversity in the locations and specific neuronal types affected, the pathology of neurodegenerative diseases is consistently linked to progressive neuronal cell death. Mitochondria are critical regulators in the apoptotic cascade, and their role in the etiology of neurodegenerative diseases is heavily substantiated in literature.

1.2.1 Mitochondrial electron transport chain and redox biology

Mitochondria are suppliers of the cells energy in the form of ATP during oxidative phosphorylation (OXPHOS). OXPHOS takes place on the folded inner membrane of the mitochondria known as the cristae. It is here that the machinery of the electron transport chain (ETC) exists in several complexes (I-V). The predominant function of the ETC machinery is to use electron transfer through the various ETC complexes to create a proton gradient across the inner membrane of the mitochondria. This gradient accounts in large part for the mitochondrial membrane potential ($\Delta\Psi_m$) which can be used as a measurement of mitochondrial health and function. The force of the proton gradient is used to drive the formation of ATP from the addition of a phosphate to ADP by ATP synthase, the final complex in the ETC.

The components of the ETC are large, multi-subunit containing complexes. These subunits must perform as individual entities and in a coordinated manner with other components for proper functioning of the ETC. A deficit in any one subunit can have catastrophic effects. The majority of the individual subunits are nuclear encoded; however, there are components of the ETC that are encoded by the mitochondrial genome. In fact, the 13 protein encoding genes of the mitochondrial genome are all components of the ETC. This is very significant, as the proper function of the ETC, and the entire oxidative phosphorylation process, is reliant on mitochondrially encoded DNA.

Complex I (NADH dehydrogenase/NADH coenzyme Q oxidoreductase) is heavily embedded in the inner membrane of the mitochondria, with a smaller portion protruding into the mitochondrial matrix. Upon binding NADH, Complex I transfers two electrons to ubiquinone Q (coenzyme Q10), forming ubiquinol and inducing a

conformational change in Complex I, resulting in a net transfer of four protons across the inner membrane to the intermembrane space. Complex III (cytochrome *c* oxidoreductase, cytochrome bc_1 complex) exists as a dimer and transfers electrons from ubiquinol to cytochrome *c*, allowing it to pump two more protons to the intermembrane space. Complex IV (cytochrome *c* oxidase) receives electrons from cytochrome *c* and transfers them to an oxygen molecule, creating two water molecules and transferring four more protons to the intermembrane space. Complex II (succinate dehydrogenase) is an independent entry point for electrons into the ETC. By oxidizing succinate, it can transfer electrons to ubiquinone to be used by Complex III. It does not transfer protons, and thus is not a direct contributor to the proton gradient. The final step in oxidative phosphorylation is the formation of ATP from ADP by Complex V (ATP synthase). This very large (600kDa) multimeric structure uses the proton gradient established by the ETC to drive the formation of ATP (Chaban et al., 2014).

The use of electron transfer to create the proton gradient is vital to the function of the ETC. Despite the highly efficient and intricately coordinated function of the ETC, it is inevitable that a subset of electrons “leak” to any electron acceptor within the system. If at any point the individual complexes of the ETC are functioning inefficiently, this electron leakage will become more substantial. The presence of electrons in such proximity to the high levels of oxygen within the mitochondria can have catastrophic effects. Indeed, mitochondria are the main contributors to cellular reactive oxygen species (ROS) production (Handy and Loscalzo, 2012). These, predominantly, are in the form of superoxide, ($O_2^{\bullet-}$) which is generated due to electron leakage from the ETC to oxygen. It is estimated that 70%-80% of cellular ROS is produced at the mitochondria,

and the majority of these are produced due to electron leakage at Complexes I or III (Handy and Loscalzo, 2012). Once formed, ROS react with and cause damage to lipids, proteins, and nucleic acids, including both nuclear and mitochondrial DNA (Cooke et al., 2003; Evans et al., 2004; Filipcik et al., 2006; Chakravarti et al., 2007). Damage to mitochondrial DNA can lead to dysfunctions in the mitochondrially encoded ETC components. This leads to a catastrophic cycle of increased electron leakage which can serve to further amplify the damage to mitochondrial proteins, lipids, and DNA, as well as other cellular components and nuclear DNA.

Due to the inevitable formation of ROS, cells have devised numerous antioxidant mechanisms to counteract this process, some of which will be highlighted here. The SOD (superoxide dismutase) family of proteins modulates the conversion of $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2). Once formed, hydrogen peroxide can be further broken down to oxygen and water by the catalase enzyme (Handy and Loscalzo, 2012). The copper/zinc-dependent SOD (SOD1) is a member of this family and is mainly located cytoplasmically, although it has been found in the mitochondria (Barber et al., 2006). This protein is of particular importance in the study of neurodegenerative diseases, as mutations are associated with familial form of amyotrophic lateral sclerosis (ALS). Another member of this family is the manganese-dependent SOD (SOD2). This protein is shown to be vital in neutralizing mitochondrial ROS (Handy and Loscalzo, 2012). Deficiencies in this protein cause early death in neonatal mice, which underscores the importance of scavenging mitochondrially produced ROS at their source (Li et al., 1995; Lebovitz et al., 1996).

Glutathione is another crucial antioxidant in various cellular compartments, including the mitochondria. This system utilizes glutathione peroxidase (GPx) and its co-factor glutathione (GSH) to convert hydrogen peroxides to water. During this reaction, glutathione (GSH) becomes oxidized to GSSG. It becomes reduced by glutathione reductase (GR) back to glutathione (GSH) (Handy and Loscalzo, 2012). Maintenance of reduced intracellular GSH levels occurs through reduction by glutathione reductase, *de novo* synthesis, or extracellular uptake (Handy and Loscalzo, 2012). Mitochondrial stores of glutathione are achieved through facilitated transport through the dicarboxylate (DIC) or 2-oxoglutarate (OGC) inner membrane carriers (Meister, 1995; Soderdahl, 2003). The importance of glutathione in regulating mitochondrial ROS is highlighted in studies which confirm that deficiency of glutathione levels indeed results in damage to the mitochondria as well as an increase in apoptosis (Meister, 1995) (discussed further in Section 1.2.3).

1.2.2 Oxidative stress and mitochondrial dysfunction in the etiology of neurodegeneration

Cells of the central nervous system (CNS) have an increased dependency on properly functioning mitochondria for energy production due to their high metabolic demands. As such, neurons are particularly sensitive to dysfunctions and impairments involving the mitochondria. This is of central importance in the etiology of neurodegenerative disorders.

Aging has long been regarded as one of the greatest risk factors for the development of neurodegenerative diseases. This is largely due to increased damage to various mitochondrial components and mitochondrial DNA (mtDNA) as aging

progresses. Due to the especially high energy demand of neurons, their susceptibility to mitochondrial dysfunction can be appreciated. Critical to this concept is the accumulation of damage to mitochondrial components and mtDNA as a result of the production of ROS (Lin and Beal 2006). As the damage to mitochondria increases, so does the production of ROS (Andreyev et al., 2005). Accordingly, mtDNA mutations linked to enhanced ROS production, including point mutations and deletions, have been found to accumulate with age, which results in a decrease in mitochondrial function and further production of ROS and mitochondrial damage (Piko et al., 1988; Cortopassi and Arnheim, 1990; Corral-Debrinski, et al., 1992; Zhang et al., 1992; Michikawa et al., 1999; Del Bo, 2002; Krishnan, et al., 2008). As this cycle progresses, it eventually leads to reduced mitochondrial membrane potential ($\Delta\Psi_m$) and failure of mitochondrial function, ultimately resulting in mitochondrial energy crisis, permeabilization of the mitochondrial outer membrane and initiation of the intrinsic apoptotic cell death cascade.

Mitochondria and neurodegeneration

Although the exact mechanisms linking aging, mitochondria, and neurodegeneration are seemingly complex, it is clear that ROS production and mitochondrial dysfunction are involved in the propagation of a catastrophic cycle which ultimately leads to induction of apoptosis and neuronal cell death. This underlying connection between mitochondria, oxidative stress, and neurodegeneration is a consistent theme found amongst neurodegenerative disorders (DiMauro and Andreu, 2000; Wallace, 2001).

Alzheimer's disease (AD), the most common form of dementia, is most prevalent in people over the age of 65. It is characterized symptomatically by a progressive

cognitive decline and histopathologically by amyloid- β ($A\beta$) peptide plaques (formed through cleavage of amyloid precursor protein (APP)), and neurofibrillary tangles containing hyperphosphorylated tau (Lin and Beal, 2006). The link between AD, mitochondrial dysfunction, and oxidative stress is heavily substantiated in literature. Damage from ROS precedes plaque formation in mouse models of AD as well as in patients exhibiting symptoms of AD (Nunomura et al., 2001; Practico et al., 2001). Furthermore, upregulation of genes linked to mitochondrial metabolism and apoptosis is also shown to occur early in the disease process (Reddy et al., 2004). Although the exact mechanistic relationship behind oxidative stress and the formation of $A\beta$ plaques and tau-fibrillary tangles is unclear, it is known that oxidative stress alters processing of both APP and tau (Lovell et al., 2004; Tamagno et al., 2005). Furthermore, expression of APP results in its localization to the mitochondrial fraction *in vitro*. Here, it induces glutathione-sensitive apoptosis, indicating that APP exerts its toxic effects, at least partially, through a direct pro-oxidant interaction with the mitochondria (Bartley et al., 2012). Finally, there is evidence that increased mtDNA damage is also involved in AD pathogenesis. It has been demonstrated that mtDNA obtained from affected regions of the brain of AD patients have as much as a 63% increase in mutations, as compared to controls (Coskun et al., 2004). Although the role of mitochondrial dysfunction in the etiology of AD is very complex and warrants further evaluation, it is evident that many aspects of mitochondrial physiology are involved in AD processes.

Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive rigidity, bradykinesia, and tremor. Its pathological hallmarks include nigral degeneration marked by cytoplasmic aggregates called Lewy bodies, which consist of α -

synuclein and ubiquitin-positive inclusions. Mitochondria have long been linked to PD etiology, as a mitochondrial complex I inhibitor, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and its metabolite MPP⁺, cause a parkinsonian phenotype in synthetic opioid users (Lin and Beal 2006). Moreover, mitochondrial complex I deficiency and glutathione depletion are observed in the substantia nigra of PD patients (Schapira et al. 1990).

In addition to mitochondrial Complex I inhibition being closely linked to the development of PD, the histopathological hallmarks of PD are shown to exist in association with mitochondrial dysfunction. Overexpression of α -synuclein, a marker of Lewy bodies, impairs mitochondrial function and increases oxidative stress in PD mouse models (Song et al., 2004). Additionally, Parkin, an E3 ubiquitin ligase whose dysfunction is linked to juvenile onset PD, has an anti-apoptotic effect through its interaction with the outer mitochondrial membrane (Darios et al., 2003; Lin and Beal 2006), whereas Parkin deficiency causes mitochondrial dysfunction and increased oxidative stress (Palacino et al. 2004; Pesah et al. 2004). As expected, oxidative damage to Parkin impairs its protective qualities and ubiquitin-ligase activities, most likely propagating a cycle of further damage (Chung et al., 2004). Finally, overexpression of glutathione *S*-transferase protects against ROS and neurodegeneration phenotypes seen in *Drosophila Parkin* mutants, further implicating that oxidative stress and mitochondrial dysfunction are significant factors in neuronal cell death (Whitworth et al., 2005). These data are consistent with the common theme in neurodegeneration of mitochondrial dysfunction being both a cause and effect in the catastrophic cycle of oxidative stress-induced cell death. Furthermore, the correlation between PD histopathological findings

and mitochondrial deficiency indicates a possible point of convergence in which this cycle becomes irreparable.

Mitochondria and Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by progressive loss of motor neurons in the cortex and spinal cord (Lin and Beal 2006). As motor neuron death ensues, progressive paralysis takes place, with patients often succumbing to respiratory failure. Approximately 10% of ALS cases are familial, resulting from inherited mutations in various genes. However, 90% of ALS cases are sporadic and are comprised of a varied and complex etiology. Importantly, in both sporadic and familial forms of ALS, post-mortem spinal cord biopsies reveal abnormalities in mitochondrial morphology and number (Lin and Beal 2006). These data provide yet another link between mitochondrial dysfunction and neuronal cell death.

In the G93A mutant SOD1 transgenic mouse model of familial ALS, mice demonstrate a defect in mitochondrial energy metabolism at disease onset in the spinal cord and brain, as compared to non-neuronal tissue (Mattiazzi et al., 2002). Under normal conditions, SOD1 is predominantly located in the cytoplasm. However, in ALS disease mouse models, mutant SOD1 becomes concentrated within mitochondrially-located vacuoles, suggesting a link between SOD1 mislocalization and ALS-associated mitochondrial deficits (Jaarsma et al., 2001; Liu et al., 2004). Additional studies demonstrate that mitochondrial localization of SOD1 occurs in concert with increased oxidative damage to mitochondrial components, impaired ATP synthesis, and diminished mitochondrial respiration (Mattiazzi et al., 2002). Importantly, disruptions in mitochondrial cristae architecture precede neuronal cell death in mice overexpressing

mutant SOD1, strongly suggesting that mitochondrial dysfunction may be a causative factor in ALS associated neuronal cell death (Kong and Xu, 1998). In addition to causing direct mitochondrial damage, mutant SOD1 also exerts negative effects through sequestering anti-apoptotic proteins, such as BCL2 (Pasinelli et al., 2004). Accordingly, mitochondrial targeting of mutant SOD1 *in vitro* results in cytochrome *c* release and subsequent apoptosis (Takeuchi et al., 2002). These data suggest that mutant SOD1 exerts its toxic effects in a two-fold fashion; first by directly interacting and affecting mitochondrial morphology and function through increased oxidative damage and ROS production, and second through disrupting anti-apoptotic proteins such as BCL2, thus effectively impeding neuronal ability to circumvent the ensuing apoptotic cascade.

1.2.3 Glutathione and neurodegeneration

Glutathione (GSH) is a necessary component of cellular antioxidant systems. It exists in various parts of the cell to exert its scavenging effects on ROS. The role of glutathione in mitochondrial redox states is one of the vital roles of this small molecule. As such, glutathione deficiencies underlie many dysfunctions of the mitochondria, and its role in the etiology of neurodegenerative disorders is heavily substantiated.

Glutathione Synthesis

Glutathione is a low molecular weight compound, whose thiol group allows it to function as a reductant. Although glutathione exists throughout many cellular compartments, its synthesis is restricted solely to the cytoplasm (Lash, 2006). Synthesis of glutathione from its precursor amino acids is ATP-dependent, and is catalyzed by γ -glutamylcysteine synthetase and GSH synthetase. The first and rate-limiting step in synthesis combines L-glutamate and L-cysteine using ATP and γ -glutamylcysteine

synthetase to form γ -glutamyl-L-cysteine. The second step involves using GSH synthetase to combine γ -glutamyl-L-cysteine and L-glycine to form the final GSH product (Lu et al., 1999). Importantly, the intracellular concentration of L-glutamate is much higher than that of L-cysteine. As such, the availability of the L-cysteine precursor largely determines the rate of glutathione synthesis (Lu et al., 1999) (Fig 1.2.4).

Glutathione Biosynthesis and Regeneration

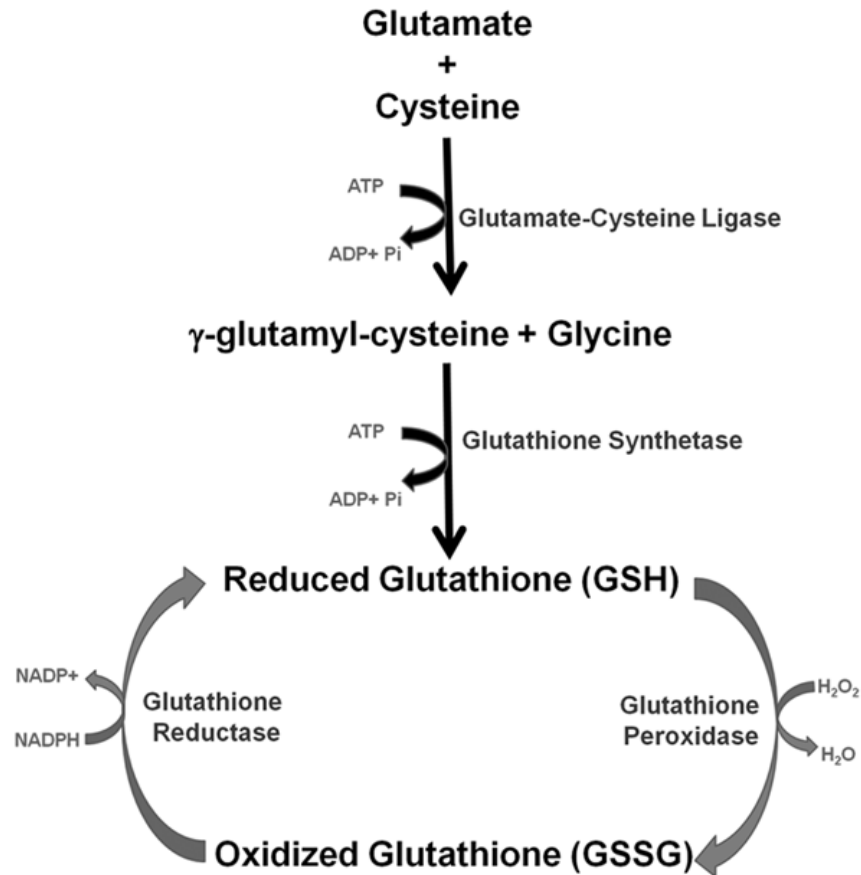


Fig. 1.2.4 – Synthesis of glutathione (GSH) is an ATP dependent process that is performed by γ -glutamylcysteine ligase and GSH synthetase combining glutamate, cysteine and glycine. Through the use of GSH peroxidase, glutathione adopts its oxidized form, GSSG, while converting H_2O_2 to water. Oxidized GSSG is converted back to its reduced form by GSSG reductase, which utilizes NADPH for this function.

Glutathione antioxidant function

A critical function of glutathione is to assist in the reduction of hydrogen peroxide (H_2O_2) produced as a result of aerobic metabolism and increased oxidative stress. By utilizing GSH peroxidase, glutathione adopts its oxidized form, GSSG, while converting H_2O_2 to water. Oxidized GSSG is converted back to its reduced form by GSSG reductase, which utilizes NADPH for this function (Lu, 1999).

Glutathione uptake into the mitochondria

Biosynthesis of GSH occurs in the cytoplasm (Griffith and Meister, 1985; McKernan et al., 1991). Therefore, GSH must be transported from the cytoplasm into the other cellular compartments once it has been synthesized. This holds true for the mitochondria, whose redox state relies heavily on the presence of GSH. Due to the negatively charged nature of GSH and the negative charge of the mitochondrial matrix, glutathione cannot passively diffuse into the mitochondria and therefore must be actively imported. Previous studies in kidney and liver elucidated that the 2-oxoglutarate (OGC; *Slc25a11*) and the dicarboxylate (DIC; *Slc25a10*) carriers of the inner mitochondrial membrane facilitated the import of glutathione (Chen and Lash, 1998; Chen and Lash 2000; Coll et al., 2003). However, less is known about neuronal import of mitochondrial glutathione. Studies indicate that inhibition of the tricarboxylate (CIC; *Slc25a1*) carrier effectively inhibits glutathione uptake in rat whole brain mitochondria (Wadey et al., 2009). Alternatively, other investigations revealed that rat cortical mitochondria are more sensitive to inhibitors of the DIC over that of the OGC. It is noteworthy that inhibition of DIC and the resultant decrease in the uptake of mitochondrial glutathione also caused a deficiency in ETC Complex I, further substantiating the importance of

mitochondrial GSH levels in maintaining proper function of mitochondrial ETC components (Kamga et al., 2010). Subsequent studies have delineated that the observed discrepancies may be due to cell-type specific variations in mitochondrial uptake. Indeed, it has been discovered that astrocyte mitochondrial glutathione levels are sensitive to both DIC and OGC inhibition, whereas cerebellar granule neuron (CGN) levels are only sensitive to DIC inhibition (Wilkins et al., 2013). Furthermore, some insight has been given into the mechanism by which mitochondrial glutathione uptake is achieved in neurons. Specifically, it has been demonstrated that BCL-2 facilitates mitochondrial uptake of GSH in rat CGNs through a direct GSH-dependent interaction with OGC (Wilkins et al., 2012).

Decreases of Glutathione in neurodegeneration

The susceptibility of neurons to mitochondrial dysfunction makes it easy to appreciate why mitochondrial glutathione levels are imperative for neuronal survival. Indeed, decreased mitochondrial glutathione levels have proven to be detrimental during the increased oxidative stress that underlies much of the mitochondrial dysfunction found in neurodegenerative disorders. Glutathione related dysfunction and deficiencies are interesting to look at in the context of PD, which is characterized by degeneration of the substantia nigra. This area of the brain is particularly prone to oxidative stress for several reasons. Dopamine (DA), which is synthesized in the substantia nigra, can be particularly toxic when produced in excess quantities. This is in large part because when not immediately taken up by post-synaptic vesicles, it is readily oxidized which can lead to the formation of superoxide and hydrogen peroxide (Hastings et al., 1996; Stokes et al., 1999). Furthermore, this area of the brain also contains very high levels of iron, which

can readily react with hydrogen peroxide via the Fenton Reaction to form hydroxyl radicals (Sofice et al., 1991). The intrinsic high propensity of the substantia nigra for oxidative stress is further compounded by the fact that this region of the brain contains relatively low levels of GSH and glutamylcysteine ligase activity (Kang et al., 1999; Pearce et al., 1997; Sian et al., 1994). Accordingly, a decrease in GSH levels correlates with increased oxidative stress in post-mortem analysis of brain tissue from PD patients (Dexter et al. 1986; Dexter et al., 1994; Sian et al., 1994; Alam et al., 1997). Studies have eluded to a potential mechanistic link between substantia nigral environment and mitochondrial complex I deficiency associated with PD. *In vitro* reduction of GSH levels specifically results in a complex I deficit (Jha et al., 2000), which provides a possible causal association between reduced GSH levels and an acknowledged component of the underlying cascade that leads to the development of PD.

Due to the preponderance of findings linking increased oxidative stress and damage to the etiology of PD, antioxidant treatment, such as glutathione supplementation, has been an appealing topic in the area of PD treatment research. However, clinical studies into GSH supplementation in PD patients have proved to be less optimistic, due to the difficulty in drug delivery across the blood brain barrier (Smeyne and Smeyne, 2013). Due to the potential therapeutic effects of GSH supplementation, research into a more effective neuronal delivery system may prove to be beneficial.

Like many neurodegenerative diseases, AD pathology is closely linked to increased neuronal oxidative damage. The main pathological hallmarks observed in AD are the formation of tau-fibrillary tangles and plaques that consist of A β peptides.

Although the toxic modalities of A β and associated plaques are seemingly complex, it has been demonstrated that A β fragments themselves cause increased oxidative stress (Hensley et al., 1994; Markesbery, 1997; Varadarajan et al., 2000; Butterfield et al., 2013). It is also demonstrated *in vitro* that the expression of A β fibrils leads to a decrease of GSH levels, which indeed propagates the production of ROS (Abramov et al., 2003; Cardoso and Oliveira, 2003).

Transgenic mouse models of AD display decreased GSH levels in hippocampal and cortical neurons prior to the onset of increased ROS levels (Ghosh et al., 2012). Interestingly, this model demonstrates an increase in the activity of glutathione peroxidase but not glutathione reductase, suggesting that the deficit may come from a failure to recycle oxidized GSSG (Resende et al., 2008). Furthermore, stimulation of GSH synthesis increases neuronal survival in mouse models of AD (Ghosh et al., 2014). Data from animal models are corroborated in post-mortem analysis of brain samples from AD patients, which indicate decreased GSH and GST levels (Lovell et al., 1998; Ramassamy et al., 2000; Venkateshappa et al., 2012). Interestingly, glutathione reductase and glutathione peroxidase mRNA levels are shown to be increased in post-mortem brain analysis of AD patients likely indicating a compensatory mechanism to offset decreased GSH levels (Aksenov et al., 1998). Although the involvement of GSH in the etiology of AD is seemingly complex, it is accepted that deficits in GSH levels and/or proteins that modulate its turnover play an important role in the pathogenesis of this disease. In fact, decreased levels of GSH can be detected in peripheral blood samples from AD patients (Bermejo et al., 2008), indicating the potential to use this as a biomarker for earlier disease diagnosis.

It is well established that increased oxidative stress and the resultant damage are principal components in the pathology of ALS. Indeed, oxidative damage to lipids, proteins and DNA has been observed in spinal cord and motor cortex of sporadic and familial forms of ALS (Shaw et al., 1995; Fitzmaurice et al., 1996; Ferrante et al., 1997). Furthermore, the involvement of the antioxidant SOD1 in familial ALS substantiates the significance of ROS in the pathogenesis of ALS. The obvious correlation between increased oxidative damage and both familial and sporadic ALS is indicative of a deficiency in antioxidant systems. Indeed, recent studies demonstrate a trend of increased oxidized glutathione in plasma of ALS patients (Baillet et al., 2010). Additionally, *in vitro* models of G93A mutant SOD1 expressing motor neurons display a decrease in GSH levels when compared to wild-type control cells (D'Alessandro et al., 2011). Studies also reveal a decrease of GSH in motor neurons of the G93A mutant hSOD1 transgenic mouse model of ALS (Rizzardini et al., 2003). These results show a potential therapeutic approach that could prove to be beneficial in treatment of ALS patients; yet, a direct link between reduction in *motor neuron* GSH levels and ALS pathology remains to be established.

1.3 Trans-activating Response DNA binding Protein/ TARDBP/ TDP-43

TDP-43 (Trans-activating response DNA binding protein, TARDBP) is a protein whose dysfunction is a hallmark of the motor neuron disease ALS and the progressive dementia disease known as frontotemporal lobar degeneration/dementia (FTLD) (Neumann et al. 2006; Cairns et al., 2007). Interestingly, a portion of both ALS and FTLD patients have overlapping symptoms as well as TDP-43 histopathological findings in upper and lower motor neuron systems, indicative of a link between these two diseases

(Lomen-Hoerth et al., 2003; Hodges et al., 2004; Neumann et al., 2006; Cairns et al., 2007; Riku et al., 2014; reviewed in Chen-Plotkin et al., 2010). Indeed, TDP-43 immunoreactive ubiquitin-positive inclusions are found in diseased tissue from both ALS and FTLN patients, indicating that these conditions may arise from a similar pathological mechanism (Cairns et al., 2006; Neumann et al., 2006; Davidson et al., 2007; Brandmeir et al., 2008). Interestingly, TDP-43 inclusions also occur in other prominent neurodegenerative diseases, including AD, PD, and HD (Amador-Ortiz et al., 2007; Hasegawa et al., 2007; Nakashima-Yasuda et al., 2007; Schwab et al., 2008; Uryu et al., 2008; Arai et al., 2009; reviewed in Chen-Plotkin et al., 2010). However, despite the preponderance of evidence supporting the role of this protein in neurodegeneration, the normal functions of TDP-43 have not been fully elucidated. Furthermore, the mechanisms of TDP-43 toxicity in ALS and FTLN are only beginning to be discovered.

TDP-43 is the main protein element of ubiquitin-positive inclusions found in most cases of sporadic and familial ALS (Neumann et al., 2006). It contains 2 RNA binding domains and a C-terminal glycine rich domain. Based on its structure, it is placed in the heterogeneous nuclear ribonuclear protein (hnRNP) family (reviewed in Chen-Plotkin, et al., 2010).

1.3.1 TDP-43 Structure and Function

The functional roles of TDP-43 have only been established in very specific, isolated contexts. TDP-43 was first discovered because of its ability to bind the transactive response region of HIV-1, where it represses gene expression (Ou et al., 1995). It is known that TDP-43 primarily exists in the nucleus, but is also continuously translocated between the nucleus and cytoplasm (Ayala et al., 2008; Winton et al., 2008).

Additional functions of TDP-43 include modulation of exon splicing (Buratti et al., 2001; Mercado et al., 2005; Ayala et al., 2008), modulation of gene expression through direct DNA and RNA binding (Ayala et al., 2005; Acharya and Reddi, 2006; Strong et al., 2007), and modulation of gene expression through stabilization of mRNA (reviewed in Chen-Plotkin et al., 2010). Recent studies have demonstrated TDP-43's involvement in gene expression through both upregulation and downregulation of select microRNAs (miRNAs), either through direct miRNA binding or binding of miRNA processing machinery (Buratti et al., 2010; Kawahara et al., 2012; Park et al., 2013, reviewed in Lee et al., 2012). Beyond its role in regulating mRNA levels, TDP-43 has also been shown to localize to cytoplasmic RNA granules, presumably indicating that it has some involvement in RNA trafficking (Wang et al., 2008). In addition to direct interactions with RNA and DNA, TDP-43 also participates in protein-protein interactions through its C-terminal glycine-rich domain, notably with other hnRNP proteins involved in alternative splicing (Buratti et al., 2005; D'Ambrogio et al., 2009). Clearly, it is evident that TDP-43 has numerous responsibilities within the cell, most of them seemingly to do with modulating some aspect of gene expression. These regulatory functions are shown to be integral in a vast array of cellular activities, from early development to metabolic pathways. As studies into the cellular functions of this protein proceed, it is likely that additional functions will become evident.

Despite the somewhat vague knowledge of TDP-43's functions, the structure of TDP-43 has been well elucidated. It contains 2 RNA binding domains (RBD) and a C-terminal glycine rich domain that is indispensable for TDP-43 gene splicing function (Ayala et al., 2005; Buratti et al., 2005). This structure has placed TDP-43 in the

heterogeneous nuclear ribonuclear protein (hnRNP) family. The N-terminus of TDP-43 contains a nuclear localization signal (NLS), consistent with its predominantly nuclear presence (reviewed in Chen-Plotkin et al., 2010; Janssens and Broeckhoven, 2013). TDP-43 also contains a leucine rich nuclear export signal (NES), which is necessary for TDP-43 cytoplasmic localization (Winton et al., 2008; Janssens and Broeckhoven, 2013). The RBDs of TDP-43 are significantly homologous to that of other hnRNP members, which is in contrast to the glycine rich domain that does not share homology (reviewed Chen-Plotkin et al., 2010) (Fig 1.3.1)

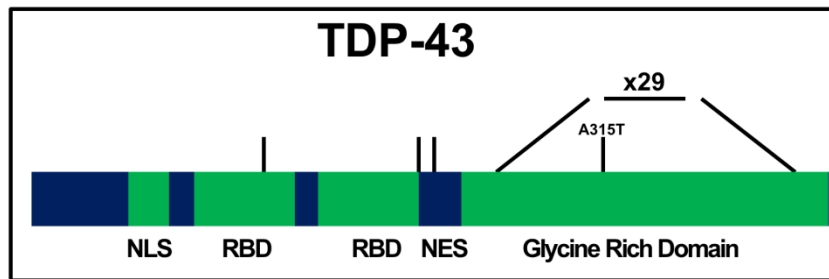


Fig. 1.3.1 - TDP-43 protein domains TDP-43 is comprised of an N-terminal nuclear localization signal (NLS) followed by two RNA-binding domains (RBD) and a centrally located nuclear export signal (NES). The glycine rich domain is located at the C-terminal end. Locations of known ALS-associated mutations are indicated by vertical lines. Of note, the majority of disease causing mutations (~29), such as the A315T mutation, are located in the C-terminal glycine rich domain.

1.3.2 TDP-43 aggregation and ubiquitination in ALS

Perhaps the most notable recent finding in regards to ALS pathology is that cytoplasmic ubiquitin-positive inclusions of TDP-43 are consistently found in *both* sporadic and most familial forms of the disease (Arai et al., 2006; Neumann et al., 2006; Davidson et al., 2007; Dickson et al., 2007; Brandmeir et al., 2008). Interestingly, TDP-43/ubiquitin-positive inclusions are not observed in familial cases involving mutant *SOD1*, indicating that disease pathology in these two subsets may arise through a different mechanism (Mackenzie et al., 2007; reviewed in Chen-Plotkin et al., 2010). The significance of these findings is underscored in that there are very few other histopathological consistencies between familial and sporadic forms of ALS. As such, evaluation of the mechanism behind dysregulation and aggregation of TDP-43 has become a recent focus in ALS research.

TDP-43/ubiquitin-positive inclusions were initially observed by Neumann et al. in brain and spinal cord motor neurons from both ALS and FTLD patients (Neumann et al., 2006). Additionally, it was demonstrated that the aggregated TDP-43 had become hyperphosphorylated and cleaved to C-terminal fragments (Arai et al., 2006; Neumann et al., 2006). Subsequent reports confirmed TDP-43 aggregation in the spinal cord and various brain regions of ALS and FTLD patients (Davidson et al., 2007; Cairns et al., 2007; Dickson et al., 2007; Brandmeir et al., 2008; Geser et al., 2008; Mori et al., 2008; Giordana et al., 2010). Moreover, some of these studies reiterate that TDP-43 inclusions are also ubiquitin-positive and/or contain phosphorylated TDP-43 (Cairns et al., 2007; Giordana et al., 2010). However, other findings indicate that not all TDP-43 positive

aggregates are necessarily ubiquitinated, indicating ubiquitination is a somewhat heterogeneous finding, and may not be a causal factor in disease pathology (Brandmeir et al., 2007; Mori et al., 2008). Although there are notable differences in the histopathology of TDP-43 aggregation, the one factor that remains consistent is that marked loss of nuclear TDP-43 occurs concomitantly with cytoplasmic localization of the protein.

Despite the preponderance of evidence demonstrating TDP-43 inclusions are an almost universal event in the pathophysiology of ALS and FTLT, the mechanism behind these events has not been as extensively clarified, and what has been discovered has varied between studies. Giordana et al. revealed that although not all TDP-43 aggregates are positive for ubiquitin, there is a fraction of soluble (not aggregated) TDP-43 that is phosphorylated, truncated and ubiquitinated (Giordana et al., 2010). In contrast, TDP-43 “pre-inclusions” (TDP-43 positive cytoplasmic granules) have been shown to lack ubiquitin-positive staining (Cairns et al., 2007; Strong et al., 2007; Giordana et al., 2010). Collectively, these data imply that although ubiquitination of TDP-43 can precede aggregate formation, it is not necessary for the formation of TDP-43 inclusions. Despite the variable results in evaluation of ubiquitination in TDP-43 inclusions, other studies indicate that C-terminal truncation and phosphorylation of TDP-43 are a more consistent finding in TDP-43 positive aggregates (Hasegawa 2008; Igaz et al., 2008; Neumann et al., 2009).

C-terminal fragments (20-25 kDa) of TDP-43 are part of the pathological hallmark of ALS. They exist in variable lengths and numbers within insoluble TDP-43 aggregates, along with full length TDP-43 (reviewed in Lee et al., 2012). The mechanism behind the generation of truncated forms of TDP-43 is not fully known.

Intriguingly, TDP-43 contains three caspase-3 consensus sequences, and is also shown to be directly cleaved into 25 and 35 kDa fragments by caspase-3 in cell culture systems (Zhang et al., 2007; Dormann et al., 2009; Zhang et al., 2009; Nishimoto et al., 2010). The involvement of caspases in TDP-43 associated neuronal cell death is a very appealing concept and gives some putative mechanistic insight into TDP-43 dysfunction. However, it remains to be evaluated whether caspase-3 is responsible for TDP-43 cleavage *in vivo*. Other possible scenarios that could render truncated forms of TDP-43 would be splice variations, cryptic transcriptional start sites (Nishimoto et al., 2010), or that other proteases (e.g., calpains) are responsible for TDP-43 cleavage. Despite the ambiguity in the mechanism behind truncation of TDP-43, it has been clearly demonstrated that these C-terminal fragments are more prone to phosphorylation than the full-length form of TDP-43, indicating that truncation of TDP-43 may be an upstream event to phosphorylation. Moreover, expression of C-terminal fragments in cell systems leads to ubiquitin-positive inclusions containing the truncated TDP-43, as well as increased apoptosis, indicating an inherent toxicity of the C-terminal fragments themselves (Igaz 2009; Zhang et al., 2009).

Along with TDP-43 truncation, phosphorylation of this protein is also found in disease associated aggregates. Of note, phosphorylation (most commonly at serine residues 409 and 410) occurs in aggregates that contain both full length and C-terminal fragments of TDP-43 (Hasegawa et al., 2008; Neumann et al., 2009). Despite these consistent findings, the mechanism of TDP-43 phosphorylation and its relationship to aggregate formation is seemingly complex. In animal models, overexpression of wild-type full length TDP-43 leads to truncated and phosphorylated TDP-43 aggregates,

suggesting that these events may be a mechanism to control protein dosage (Xu et al., 2010). Moreover, phosphorylated C-terminal fragments of TDP-43 appear to be more resistant to proteosomal degradation than non-phosphorylated fragments, indicating that a potential mechanism behind aggregate formation may be through a slowed clearance by cellular degradation machinery (Zhang et al., 2010). Although studies confirming the presence of phosphorylated TDP-43 in disease models are repeatedly substantiated, evaluation of the toxic effects of phosphorylation elicits a more complex picture. Mutation of TDP-43 phosphorylation sites to non-phosphorylatable forms in *mutant*-TDP-43 expressing *C. elegans* models rescues neurotoxic phenotypes, while mutation of phosphorylation sites of WT TDP-43 has little to no effect (Liachko et al., 2010). Conversely, expression of non-phosphorylatable TDP-43 in mammalian cells has little effect on TDP-43 aggregation while, expression of phospho-mimetic forms of TDP-43 reveals a dramatic reduction in TDP-43 aggregation. This is surprising in that it signifies a protective effect TDP-43 phosphorylation (Brady et al., 2011). In accord with the variation in data surrounding ubiquitination and truncation of TDP-43, phosphorylation also appears to have a complex involvement in TDP-43 aggregate formation. Although it is accepted that these two events are tightly coupled, the underlying mechanisms and effects of TDP-43 phosphorylation and aggregate formation remain to be determined.

1.3.3 TDP-43 Mutations in ALS

Since the discovery of TDP-43 protein inclusions in most cases of ALS, there has been an emerging agreement that TDP-43 is mechanistically linked to ALS pathology. Accordingly, many mutations in TDP-43 have been discovered that are linked to both sporadic and familial forms of ALS (Pesiridis et al., 2009). Familial ALS associated

TDP-43 mutations are inherited in an autosomal dominant manner (Pesiridis et al., 2009). Importantly, the majority of disease-associated mutations are found in the glycine-rich domain, further signifying the importance of this region in TDP-43 function (Kovacs et al., 2009). To date, 29 different disease causing missense mutations have been documented, 28 of which are located in the C-terminal glycine-rich domain. These mutations are found to occur in both familial and sporadic forms of ALS, as well as in cases of FTLN, further highlighting the mechanistic link between these two diseases (Sreedharan et al., 2008; Benajiba et al., 2009; Pesiridis et al., 2009). Importantly, TDP-43 mutations are correlated with TDP-43 histopathological hallmarks in ALS patients, further supporting a correlation between protein dysfunction and ALS disease pathology (Pesiridis et al., 2009). Although there are limited data into effects of TDP-43 mutations, a notable finding is that mutations of another hnRNP protein, fused in sarcoma (FUS), are also linked to familial forms of ALS. This further substantiates a causal link between dysfunctional RNA regulation and the pathogenesis of ALS, and perhaps provides some insight into the mechanism of toxicity of TDP-43 mutations (Kwiatkowski et al., 2009).

The Ala-315-Thr (A315T) mutation in TDP-43 was initially discovered in a genetic screen of families exhibiting an autosomal dominant inheritance of ALS (Glitcho et al., 2008). This residue is highly conserved within the glycine-rich domain, indicative of a functional importance. An evaluation of spinal cord samples from A315T TDP-43 patients reveals significant TDP-43 histopathology concomitant with extensive neuronal loss. These findings are consistent with what is observed in other ALS cases (Cairns et al., 2010). Unfortunately, studies into the effects of this mutation in mouse models yield a somewhat convoluted story. Wegorzewska et al. (2009) reported that the A315T

mutant overexpressing mice developed a neuronal pathology that is reminiscent of ALS. In contrast, subsequent studies using this mouse model have been less successful, due to the mice succumbing to gastrointestinal inclusions prior to any neurological phenotype (Esmaeili et al., 2013; Herdewyn et al., 2014). Although, complete characterization of this mouse model has not occurred, *in vitro* studies reveal that overexpression of mutant A315T constructs (but not WT TDP-43) in primary motor neuronal cultures is toxic and results in TDP-43 inclusions that are consistent with ALS histopathology (Kabashi et al., 2010). However, the effects of A315T TDP-43 expressed at physiological levels remain to be determined. Based on the link between mutant TDP-43 and familial ALS the importance of further evaluation of the effects of this mutation can be appreciated.

1.3.4 Loss of Function verses Gain of Function and TDP-43 Toxicity

The involvement of TDP-43 dysfunction in ALS is perhaps the most consistent pathological finding in a disease spectrum that is otherwise highly variable. Like other neurodegenerative diseases that carry hallmark cellular pathologies, the cause and effect relationships between TDP-43 histopathology and ALS disease etiology is seemingly complex and remains to be definitively characterized. A central challenge to unraveling the mechanism involved in TDP-43 mediated toxicity is to ascertain whether toxic effects occur in a gain of function (GOF) or loss of function (LOF) manner. Indeed, current literature supports both options, and a dichotomy remains on the answer to this question.

TDP-43 has been demonstrated to have a role in various stages of gene expression through its RNA/DNA binding properties as well as through protein-protein interactions. However, these findings have occurred in isolated contexts and are seemingly very specific to given cell types. Furthermore, as new data emerges, it is apparent that TDP-

43 function likely extends far beyond what is currently described. As such, ascertaining TDP-43 LOF effects, especially *in vivo*, has proven to be especially challenging. Nonetheless, there is evidence supporting that this may be a mechanism behind TDP-43 mediated-toxicity.

Perhaps the strongest indicator that TDP-43 toxicity occurs through LOF effects is the observation that TDP-43 cytoplasmic inclusions occur concomitantly with nuclear clearing of the protein (Neumann et al., 2006). This is significant in that TDP-43 has been shown to exert many of its gene expression effects within the nucleus, such as, DNA binding and modulation of RNA splicing. ALS associated decreases in nuclear TDP-43 levels would almost certainly render TDP-43 non-functional in these roles. Accordingly, *in vitro* evidence suggests that even through overexpression of full length and C-terminal TDP-43 fragments, TDP-43 aggregation occurs concomitantly with a disruption in previously established TDP-43 splicing abilities (Igaz et al., 2009). This indicates that TDP-43 function is in fact impaired under conditions that replicate ALS associated TDP-43 pathology. Interestingly, a recent study demonstrates that expression of C-terminal TDP-43 fragments results in TDP-43 aggregates that also contain RNA Polymerase II as well as transcription factors Sp1 and CREB, suggesting that the effects of loss of function extend beyond that of TDP-43 (Yamashita et al., 2014). Although it remains to be determined, it is an intriguing possibility that additional factors are sequestered within TDP-43 inclusions. An important point to be made is that protein sequestration opens up the possibility of GOF toxicity from TDP-43 cytoplasmic aggregates. These findings are especially intriguing in that they represent a convergence point in LOF and GOF.

TDP-43 GOF toxicity theories have been significantly investigated as a causal factor in ALS-associated neuronal cell death. This is due, in large part, to the autosomal dominant inheritance pattern of TDP-43 mutant linked ALS. Furthermore, the majority of studies that recapitulate ALS-associated TDP-43 histopathological features, both *in vitro* and *in vivo*, are based on overexpression of either WT or mutant forms of TDP-43. The central premise of these theories is that TDP-43 aggregates themselves (or cytoplasmic localization) exert neuronal toxicity. Another possibility is that TDP-43 toxicity occurs through actions in the cytoplasm that would not normally occur, or would occur less substantially, if TDP-43 remained primarily nuclear. Moreover, aberrant TDP-43 phosphorylation, as is observed in ALS, could theoretically cause atypical TDP-43 functions that would have detrimental cellular effects. As with LOF theories, *in vivo* and *in vitro* studies are in the early stages of delineating possible GOF effects of TDP-43 proteinopathy.

It is well established that overexpression of TDP-43 C-terminal fragments form toxic cytoplasmic aggregates that mimic ALS-associated TDP-43 histopathology. However, investigations into the mechanism of toxicity have yielded variable results. It is recently described that overexpression of C-terminal fragments of TDP-43 in HEK cells does not affect the cellular distribution of endogenous TDP-43, even though TDP-43 cytoplasmic aggregates are formed. This suggests that the toxic effects are not due to loss of nuclear TDP-43 function in this model (Zhang et al., 2009). However, this is in contrast to studies in neuronal-like NSC-34 cells, demonstrating a loss of nuclear TDP-43 upon overexpression of C-terminal fragments (Yang et al., 2010), suggesting that results are dependent on the experimental systems used. Interestingly, investigations in primary

neuronal cultures indicate that TDP-43 mediated toxicity is not dependent on aggregate formation. Rather, increased cytoplasmic levels of non-aggregated TDP-43 are a better indicator of cellular toxicity than decreased nuclear levels (Barmada et al., 2010). This is suggestive that toxicity occurs through aberrant TDP-43 functions in the cytoplasm. It is also demonstrated that TDP-43-mediated toxicity is dependent on RNA-binding activity (Voigt et al., 2010), further suggesting that toxicity may be caused by a rogue TDP-43 function.

As with other areas of TDP-43 investigations, the LOF and GOF debate remains a central component of current ALS research. Clearly, evidence exists that supports both possibilities. TDP-43 dysfunction occurs through seemingly complex and variable mechanisms, and it is equally likely that TDP-43 mediated toxicity occurs in a very complex manner. This alludes to the possibility that both LOF and GOF effects are involved. However, a more thorough understanding will be possible as TDP-43 functions become fully elucidated.

**Chapter Two: N-terminal cleavage of the mitochondrial fusion GTPase OPA1
occurs via a caspase-independent mechanism in cerebellar granule neurons exposed
to oxidative or nitrosative stress**

2.1 Abstract

Neuronal cell death via apoptosis or necrosis underlies several devastating neurodegenerative diseases associated with aging. Mitochondrial dysfunction resulting from oxidative or nitrosative stress often acts as an initiating stimulus for intrinsic apoptosis or necrosis. These events frequently occur in conjunction with imbalances in the mitochondrial fission and fusion equilibrium, although the cause and effect relationships remain elusive. Here, we demonstrate in primary rat cerebellar granule neurons (CGNs) that oxidative or nitrosative stress induces an N-terminal cleavage of optic atrophy-1 (OPA1), a dynamin-like GTPase that regulates mitochondrial fusion and maintenance of cristae architecture. This cleavage event is indistinguishable from the N-terminal cleavage of OPA1 observed in CGNs undergoing caspase-mediated apoptosis (Loucks et al., 2009) and results in removal of a key lysine residue (K301) within the GTPase domain. OPA1 cleavage in CGNs occurs coincident with extensive mitochondrial fragmentation, disruption of the microtubule network, and cell death. In contrast to OPA1 cleavage induced in CGNs by removing depolarizing extracellular potassium (5K apoptotic conditions), oxidative or nitrosative stress-induced OPA1 cleavage caused by complex I inhibition or nitric oxide, respectively, is caspase-

independent. N-terminal cleavage of OPA1 is also observed *in vivo* in aged rat and mouse midbrain and hippocampal tissues. We conclude that N-terminal cleavage and subsequent inactivation of OPA1 may be a contributing factor in the neuronal cell death processes underlying neurodegenerative diseases, particularly those associated with aging. Furthermore, these data suggest that OPA1 cleavage is a likely convergence point for mitochondrial dysfunction and imbalances in mitochondrial fission and fusion induced by oxidative or nitrosative stress.

2.2 Introduction

It is increasingly clear that aging and neurodegeneration are mechanistically linked through oxidative or nitrosative stress, mitochondrial dysfunction, and disruption of the mitochondrial fission and fusion equilibrium. Aging is often reported as the greatest risk factor for the onset of sporadic neurodegenerative diseases and this correlation is largely attributed to mitochondrial dysfunction (Bossy-Wetzel et al., 2003 and Lin and Beal, 2006). However, the mechanistic interrelationships between oxidative or nitrosative stress, mitochondrial dysfunction, and alterations in mitochondrial fission and fusion are complex and not well defined. Therefore, identifying molecular events which link two or more of these factors to neuronal cell death will help unravel the pathological basis of the association between neurodegeneration and aging.

The aberrant production of ROS and reactive nitrogen species (RNS), or deficits in endogenous antioxidant or free radical scavenging systems, appear to be critical factors in neurodegenerative disorders and aging. Evidence indicates that increased oxidative or nitrosative damage through both ROS and RNS in the substantia nigra likely plays a

significant role in dopaminergic cell death in PD (Jenner, 2003). In particular, complex I inhibition and the associated mitochondrial oxidative stress are pathogenic in PD and have led to the development of several neurotoxin models of this disease including MPTP and rotenone (Przedborski and Vila, 2003 and Sherer et al., 2007). Studies of post-mortem AD brain tissue also reveal the adverse effects of ROS and RNS, including lipid peroxidation, increased protein carbonyls, and enhanced peroxynitrite-mediated damage (Smith et al., 1997; Lin and Beal, 2006). Similarly, increased protein carbonyls, 3-nitrotyrosine, and lipid peroxidation have been observed in spinal cord motor neurons and CSF of patients with amyotrophic lateral sclerosis (ALS) (Barber and Shaw, 2010). Importantly, increased oxidative damage as a result of mitochondrial dysfunction has also been demonstrated in aging, indicating a potential pathway where aging and neurodegeneration converge (Bossy-Wetzel et al., 2003).

Mitochondria are essential organelles that participate in diverse functions such as ATP production, buffering of intracellular calcium, maintenance of cell survival through sequestration of cytochrome *c*, and apoptotic signaling through the release of proapoptotic factors (Ow et al., 2008; Sheridan and Martin, 2010). Mitochondria exist in a dynamic state and their proper function relies on a critical balance of fission and fusion events (Detmer and Chan, 2007). Impairment of this delicate balance can lead to decreased mitochondrial respiration rates and increased ROS production, leading to a catastrophic cycle of further damage and dysfunction (Chen et al., 2005 and Knott et al., 2008). Neurons are particularly susceptible to impairments in mitochondrial dynamics due to their increased energy demands (Detmer and Chan, 2007; Knott et al., 2008).

Accordingly, a fundamental link between mitochondrial impairment and neurodegeneration is heavily substantiated by current literature (Perry et al., 2002; Lin and Beal, 2006).

Several proteins of the dynamin family of large GTPases are responsible for mitochondrial fission and fusion, and disruptions of the fission and fusion machinery have been linked to neurodegeneration (Knott et al., 2008; Cho et al., 2010). Optic atrophy-1 (OPA1) is a member of this GTPase family and is a major player in fusion of the inner mitochondrial membrane and maintenance of cristae architecture, events which are orchestrated through oligomerization of differentially processed forms of OPA1. Oligomerization of OPA1 is necessary for sequestration of cytochrome *c* in the mitochondrial intermembrane space, and disruption in this function is shown to induce the intrinsic apoptotic cascade (Olichon et al., 2003; Cipolat et al., 2004). OPA1 is highly expressed in retinal tissue during development and into adulthood (Aijaz et al., 2004). The importance of proper OPA1 function is underscored by its involvement in the neurodegenerative condition, autosomal dominant optic atrophy type 1, which involves mutations in the gene encoding for OPA1 (Delettre et al., 2000). Individuals with this disease develop progressive blindness as a result of degeneration of retinal ganglion cells and their axons that comprise the optic nerve. A subset of patients exhibit additional multi-system neurological deficits as a result of mitochondrial impairment (Amati-Bonneau et al., 2009). *In vitro* studies to delineate possible mechanisms by which OPA1 mutations cause disease have indicated that down regulation or loss-of-function of OPA1 results in mitochondrial fragmentation and disruption of the cristae architecture.

Although the importance of OPA1 expression in retinal tissue is pathologically relevant, additional neuronal expression of OPA1 has been demonstrated in the motor cortex, the frontal brain, the spinal cord, and the cerebellar cortex (Delettre et al., 2001; Misaka et al., 2002; Aijaz et al., 2004). The importance of OPA1 expression in neurological function is evident, yet its expression in non-neural tissue indicates a widespread function.

OPA1 exists as eight alternatively-spliced variants (Delettre et al., 2001). Upon import into the mitochondria, the mitochondrial targeting sequence is cleaved by mitochondrial processing peptidases to produce a long (*L*) isoform (Ishihara et al., 2006). Within the mitochondria, OPA1 undergoes further cleavage at several positions to yield soluble, short (*S*) isoforms. Here, processing of OPA1 becomes more complex. OPA1 is constitutively cleaved into a short isoform by the ATP-dependent metalloprotease YME1L1 (Griparic et al., 2007; Song et al., 2007). Additional cleavage events are mediated by the *m*-AAA proteases paraplegin and AFG3L1 -2 during loss of mitochondrial membrane potential, and the presenilin-associated rhomboid-like (PARL) protease during apoptosis (Cipolat et al., 2006; Song et al., 2007; Guillery et al., 2008). The overlap in actions of these proteases indicates a potential redundancy in their functions; this is highlighted by the observation that effective siRNA knock-down of any one of these proteases does not abolish OPA1 processing (Griparic et al., 2007; Guillery et al., 2008).

Differential processing of OPA1 splice variants results in at least five distinct isoforms (denoted “a”–“e”), all of which are visible by Western blotting. We previously

reported the appearance of a novel, smaller OPA1 cleavage product (denoted “f”) upon induction of apoptosis in primary cultures of rat cerebellar granule neurons (CGNs). This N-terminal cleavage event removes a critical portion of the GTPase domain including K301 and, therefore, GTPase activity would be lost and OPA1 rendered non-functional (Loucks et al., 2009). This is evident in the observed fragmentation of the mitochondrial network coincident with N-terminal OPA1 cleavage. In CGNs, the N-terminal OPA1 cleavage induced during apoptosis was demonstrated to be caspase-dependent, yet *in vitro* experiments revealed that caspases were not able to directly cleave OPA1. Our current studies reveal that this N-terminal OPA1 cleavage also occurs during oxidative or nitrosative stress-induced neuronal cell death, but in a caspase-*independent* manner. This particular cleavage event has yet to be identified within a specific neurodegenerative condition; however, we demonstrate that N-terminal cleavage of OPA1 occurs spontaneously with advancing age in rat and mouse midbrain and hippocampal tissues. These findings further connect mitochondrial dysfunction to aging, a principle risk factor for neurodegeneration.

2.3 Materials and Methods

2.3.1 Materials

The monoclonal antibody to OPA1 (residues 708–830) was obtained from BD Biosciences (San Diego, CA, USA). The polyclonal antibody to the C-terminus of OPA1 (residues 947–960) was prepared as described by Zhu et al. (2003). The polyclonal antibody to actin was from Abcam (Cambridge, MA, USA). Horseradish peroxidase-

linked secondary antibodies and reagents for enhanced chemiluminescence were from GE Healthcare (Pittsburgh, PA, USA). MitoTracker Green used to detect mitochondria was obtained from Invitrogen (Grand Island, NY, USA). The monoclonal β -tubulin antibody, MPP⁺, the pan-caspase inhibitor BOC-D (OMe)-FMK and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO, USA). A CY3-conjugated secondary antibody for immunofluorescence was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Rotenone, sodium nitroprusside (SNP), JNK inhibitor II SP600125, p38MAP kinase inhibitor SB 203580, QVD, and ALLN were from CalBiochem (San Diego, CA, USA).

2.3.2 Cell Culture

Primary rat cerebellar granule neurons (CGNs) were prepared as described previously (Loucks et al., 2009). Briefly, CGNs were isolated from 7-day-old Sprague-Dawley rat pups of both sexes, with the addition of cytosine arabinoside (10 μ M) after 24 h *in vitro* to inhibit non-neuronal cell growth. Experiments were performed after 6–7 days *in vitro* (DIV).

2.3.3 Brain tissue preparation

Rat midbrain, hippocampal, cerebellum and substantia nigra tissues were dissected from 4 month-old and 22 month-old Sprague-Dawley rats and immediately dounce-homogenized in lysis buffer. Protein lysates were obtained and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through 7.5% polyacrylamide gels. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham) and immunoblotted as described in the results section.

Mouse midbrain, hippocampal, cerebellum and cortical tissues were dissected from 4 month-old and 15 month-old female FVB mice. Tissues were homogenized and protein lysates obtained as described for rat tissues.

2.3.4 Cell lysis and immunoblotting

After treatment as described in the Results section, cells were washed once in 1 mL ice-cold phosphate-buffered saline (PBS) (pH 7.4). Cells were incubated for 10 min on ice in lysis buffer (150–200 μ l per 35-mm well) prepared as described previously (Loucks et al., 2006). The cells were then harvested by scraping and cell debris were removed by centrifugation at 6000 g for 2 min. Protein concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) with final absorbance of samples measured by a microplate spectrophotometer (BioTek PowerWave XS2, Winooski, VT, USA). Whole cell lysates were subjected to SDS-PAGE through 7.5% polyacrylamide gels. Resolved proteins were transferred to PVDF membranes and processed for immunoblot analysis as previously described (Loucks et al., 2006). In general, western blots shown are representative of a minimum of three independent experiments.

2.3.5 Immunofluorescence/mitochondrial staining

After treatment as described in the Results section, cells were fixed for 1 h at room temperature in 4% paraformaldehyde followed by permeabilization and blocking in 0.2% Triton X-100 and 5% BSA in 1X PBS (pH 7.4). To obtain microtubule staining, β -tubulin primary antibody was diluted 1:500 in 2% BSA and 0.2% Triton X-100 in PBS. Cells were incubated in the primary antibody for approximately 16 h (overnight) at 4 °C

and subsequently washed five times in 1X PBS and placed in CY3-conjugated secondary antibody and DAPI diluted in 2% BSA and 0.2% Triton X-100 in PBS. Cells were then washed five times in 1X PBS followed by addition of anti-quench (5 mg/ml *p*-phenylenediamine in 1X PBS). To obtain mitochondria staining, CGNs were treated as described in the Results section and subsequently rinsed in Hanks Balanced Salt Solution (HBSS). Cells were incubated for 30 min at 37 °C, 10% CO₂ in MitoTracker Green dye and Hoechst stain diluted in HBSS followed by a HBSS rinse. Fluorescent images of both β -tubulin and MitoTracker staining were captured using a Zeiss Axioplan 2 fluorescence microscope equipped with a Cooke Seniscam CCD camera and Slidebook image analysis software (Intelligent Imaging Innovations, Inc., Denver, CO, USA). Five images per well were captured and three experiments were completed per treatment.

2.4 Results

2.4.1 5K-induced CGN apoptosis triggers caspase-dependent N-terminal OPA1 cleavage

We have previously revealed a novel N-terminal cleavage of OPA1 which is induced by various apoptotic stimuli (Loucks et al., 2009). Specifically, OPA1 cleavage is observed in CGNs upon removal of depolarizing potassium and serum (5K conditions) for 24 h (Loucks et al., 2009) (Fig. 2.4.1 A; cleavage product is denoted as band “f”). Consistent with our previous results, this cleavage product is detectable by Western blotting using a monoclonal antibody targeted to an epitope in the middle of the OPA1 sequence (BD OPA1). Next, we examined OPA1 cleavage using a polyclonal antibody directed against the last 14 C-terminal residues. The OPA1 cleavage product is also

detected with the C-terminal antibody, indicating that the cleavage event occurs at the N-terminus of OPA1 (Fig. 2.4.1B). Furthermore, OPA1 cleavage induced under 5K conditions was substantially reduced upon incubation with the broad spectrum caspase inhibitors BOC and QVD (Fig. 2.4.1 A, B). These results support our previous findings that N-terminal cleavage of OPA1 occurs via a caspase-dependent mechanism during induction of CGN apoptosis via removal of depolarizing potassium.

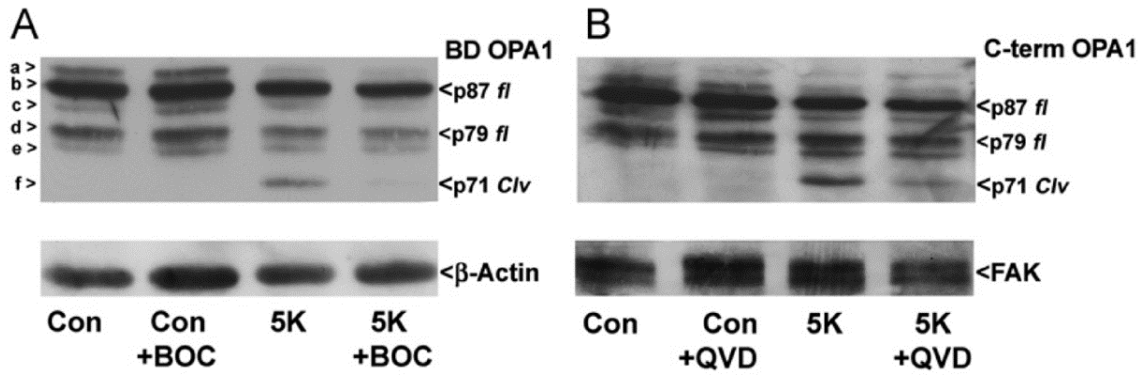


Fig. 2.4.1 - Removal of depolarizing extracellular potassium (5K) induces N-terminal cleavage of OPA1 that is caspase-dependent. (A–B) CGNs were incubated for 24 h in either control medium (25K+serum) or medium lacking serum and containing only 5 mM KCl (5K) +/- broad spectrum caspase inhibitor BOC (10 μ M) (A) or QVD (10 μ M) (B). OPA1 cleavage (Clv) was detected using a monoclonal antibody that detects residues 708–830 of OPA1 (BD OPA1) (A) or a polyclonal antibody against the C-terminal 14 amino acids, residues 947–960 (C-term) (B). The blots were then stripped and reprobed for β -Actin (A) or FAK (B) as loading controls. OPA1 isoforms a–f are indicated to the left in (A). p87 *fl* and p79 *fl* indicate full length OPA1 isoforms. p71 *clv* denotes the cleavage product.

2.4.2 Mitochondrial fragmentation occurs in concert with N-terminal cleavage of OPA1.

We previously established that the N-terminal cleavage of OPA1 described above removes a key residue, K301, which is critical for the GTPase function of OPA1. This was determined by performing a size comparison of the OPA1 cleavage product with a PCR generated deletion mutant lacking the N-terminal 300 amino acids (Loucks et al., 2009). To further examine the loss of functionality of OPA1 after N-terminal cleavage, we investigated whether 5K treatment elicits increased mitochondrial fragmentation. Mitochondria adopt a rounded and fragmented morphology when CGNs are incubated in 5K for 24 h, as visualized by MitoTracker Green staining. This is in contrast to the tubular and interconnected mitochondrial morphology that is characteristic of control cells (Fig. 2.4.2 A, B). To further assess cell morphology during induction of apoptosis, we evaluated fragmentation of the microtubule network, as visualized by β -tubulin immunostaining. CGNs incubated in 5K for 24 h displayed a slight increase in fragmentation of the microtubule network, demonstrated by the appearance of punctate foci. This is in contrast to the interconnected microtubule network observed in control cells (Fig. 2.4.2 C, D). However, the microtubule network of 5K-treated cells did not display the degree of fragmentation observed in the mitochondrial networks, indicating that the observed mitochondrial fragmentation was not a result of complete breakdown of cytoskeletal architecture.

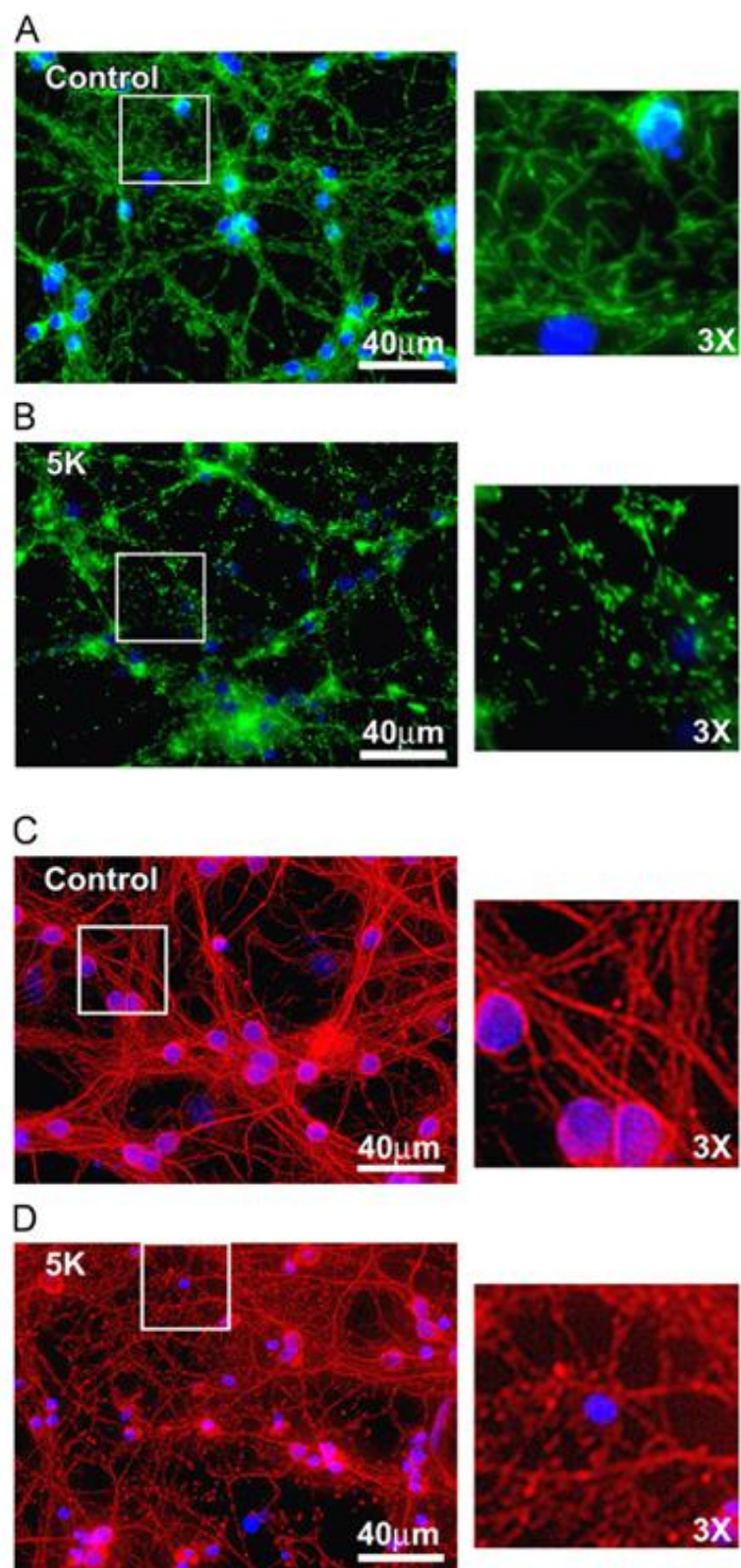


Fig. 2.4.2 - CGNs display significant mitochondrial fragmentation but minimal microtubule disruption following 5Ktreatment. (A–B) CGNs were treated as in Fig.1. Live imaging was performed using MitoTracker to detect mitochondria (green) and Hoechst to detect nuclei (blue). (C–D) CGNs were treated as in (A–B), fixed and immunostained for β -Tubulin (red). Nuclei were detected with DAPI (blue).

2.4.3 Complex I inhibition with MPP⁺ induces caspase-independent N-terminal cleavage of OPA1 that is otherwise indistinguishable from that observed during 5K-induced apoptosis

Oxidative stress is implicated as a prominent mechanism of mitochondrial dysfunction and neuronal cell death associated with neurodegeneration. We sought to determine the effects of various oxidative stressors on the cleavage of OPA1. Initially, we exposed CGNs to 1-methyl-4-phenylpyridinium (MPP⁺), a potent inhibitor of complex I of the ETC often used to model PD. We have previously shown that MPP⁺ induces CGN death independent of caspase activation (Harbison et al., 2011). Following MPP⁺ treatment, an OPA1 cleavage product was observed that was indistinguishable from that produced by 5K. The MPP⁺-induced OPA1 cleavage product was detected by both the BD monoclonal and C-terminal specific OPA1 antibodies (Fig. 2.4.3A, B). However, in contrast to the caspase-dependent cleavage of OPA1 induced by 5K treatment, OPA1 cleavage resulting from MPP⁺ treatment was insensitive to the pan-caspase inhibitors BOC and QVD (Fig. 2.4.3C). This indicates that OPA1 cleavage induced by oxidative stress occurs in a *caspase-independent* manner, via a mechanism distinct from that observed under classically apoptotic conditions (*i.e.*, 5K). Additionally, OPA1 cleavage induced by MPP⁺ treatment was not inhibited by co-treatment with the calpain inhibitor ALLN (Fig. 2.4.3D), indicating that activation of the protease calpain is not a significant factor in the N-terminal cleavage of OPA1. To define a possible role for OPA1 cleavage in oxidative stress-induced CGN death, we compared the timing of OPA1 cleavage to cell death induced by MPP⁺. OPA1 cleavage was detectable by Western blotting after 10 h of MPP⁺ treatment (Fig. 2.4.3E). By comparison, nuclear condensation was first apparent

after 8 h of treatment, with significant nuclear condensation occurring after 12 h of exposure to MPP^+ (Fig. 2.4.3 F). These data indicate that N-terminal OPA1 cleavage likely forms part of the cell death cascade and is not merely an end result of overt cell death induced by MPP^+ .

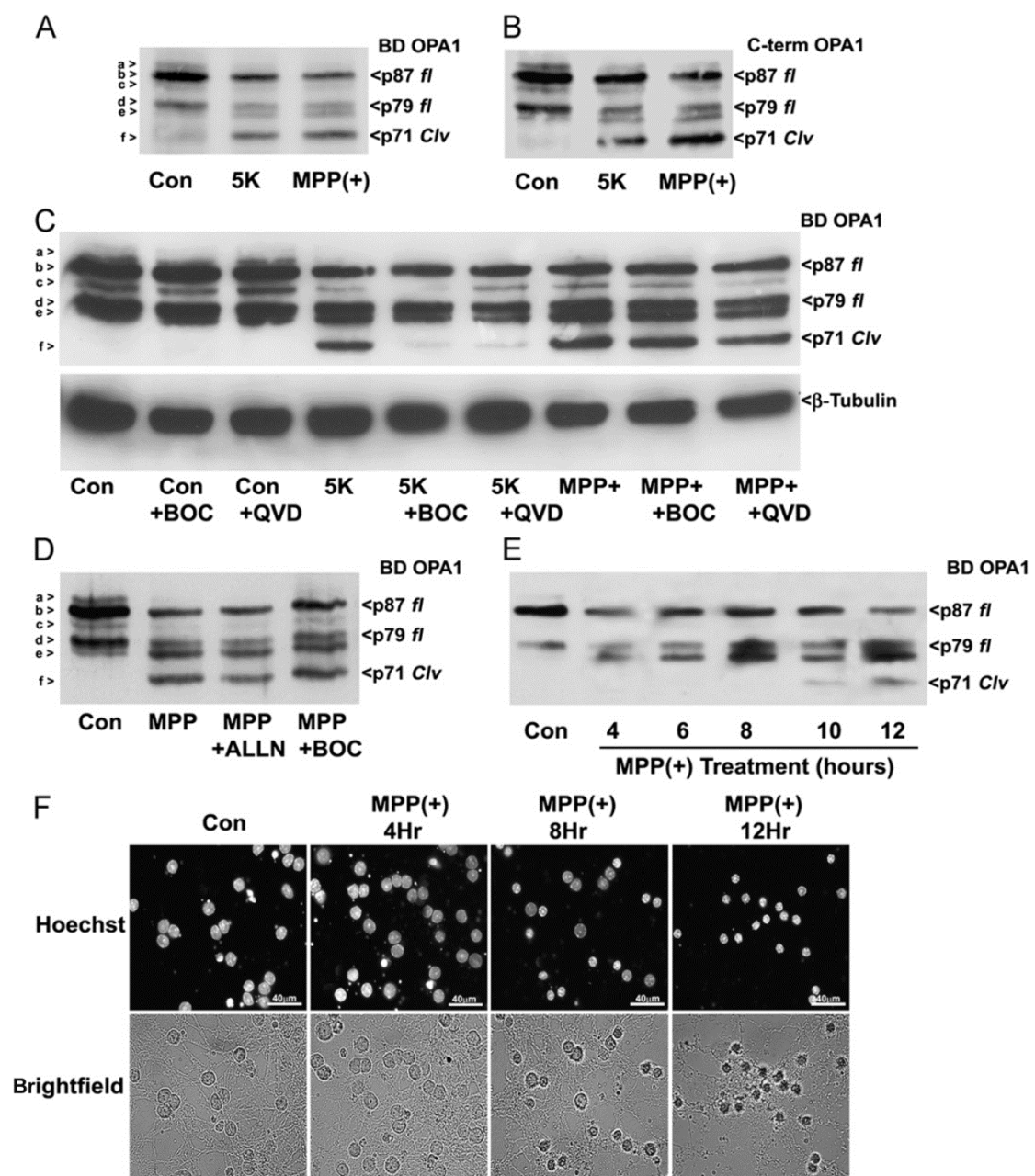


Fig 2.4.3 - OPA1 cleavage induced by the complex I inhibitor, MPP⁺, is indistinguishable from 5K-induced OPA1 cleavage but is caspase-independent.(A–B)

CGNs were incubated for 24 h in either control medium (25K+serum), 5K, or medium containing the complex I inhibitor MPP⁺ (150 μ M). OPA1 cleavage (Clv) was detected by Western blotting using a monoclonal antibody that detects residues 708–830 of OPA1 (BD OPA1) (A) or an antibody that detects the C-terminal 14 amino acids (B). (C) CGNs were incubated in either control medium (25K+serum), 5K, or medium containing 150 μ M MPP⁺ as in (A) +/- pan-caspase inhibitors BOC (10 μ M) or QVD (10 μ M). OPA1 cleavage was detected by Western blotting with a monoclonal antibody to residues 708-830 of OPA1 (BD OPA1). The blot was then stripped and reprobed for β -Tubulin as a loading control. (D) CGNs were treated with MPP⁺ (150 μ M) for 24 h +/- the calpain inhibitor ALLN or the pan-caspase inhibitor BOC (10 μ M). OPA1 cleavage was detected as in (A). (E–F) MPP⁺-induced OPA1 cleavage occurs concurrently with nuclear condensation. CGNs were incubated for up to 24 h in medium containing MPP⁺ (150 μ M), with OPA1 cleavage evaluated by Western blotting as in (A) at 0, 4, 6, 8, 10, and 12 h of treatment. (F) CGNs were incubated in medium containing MPP⁺ (150 μ M) and nuclear morphology was evaluated using either bright-field microscopy or Hoechst stain to detect nuclei at 0, 4, 8 and 12 h of treatment. OPA1 isoforms a–f are indicated to the left of each Western blot. p87 *fl* and p79 *fl* indicate full length OPA1 isoforms. p71 *clv* denotes the cleavage product.

2.4.4 MPP⁺ induces striking mitochondrial fragmentation and complete disruption of the microtubule network in CGNs

We next investigated whether MPP⁺ treatment had an effect on mitochondrial fragmentation similar to 5K treatment. MPP⁺ treatment rendered the mitochondrial network of CGNs completely fragmented, with areas of small punctate foci in addition to areas of larger mitochondrial aggregates. This change in mitochondrial structure was striking in comparison to the interconnected mitochondrial network in control cells (Fig. 2.4.4A, B). The effects of MPP⁺ on the mitochondrial network appeared more severe than those observed with 5K treatment. In addition, the microtubule network was significantly more fragmented with MPP⁺ than what was observed during 5K treatment, as revealed by very diffuse β -tubulin staining, along with the appearance of tubulin aggregates rather than distinct microtubules as seen in control CGNs (Fig. 4C, D).

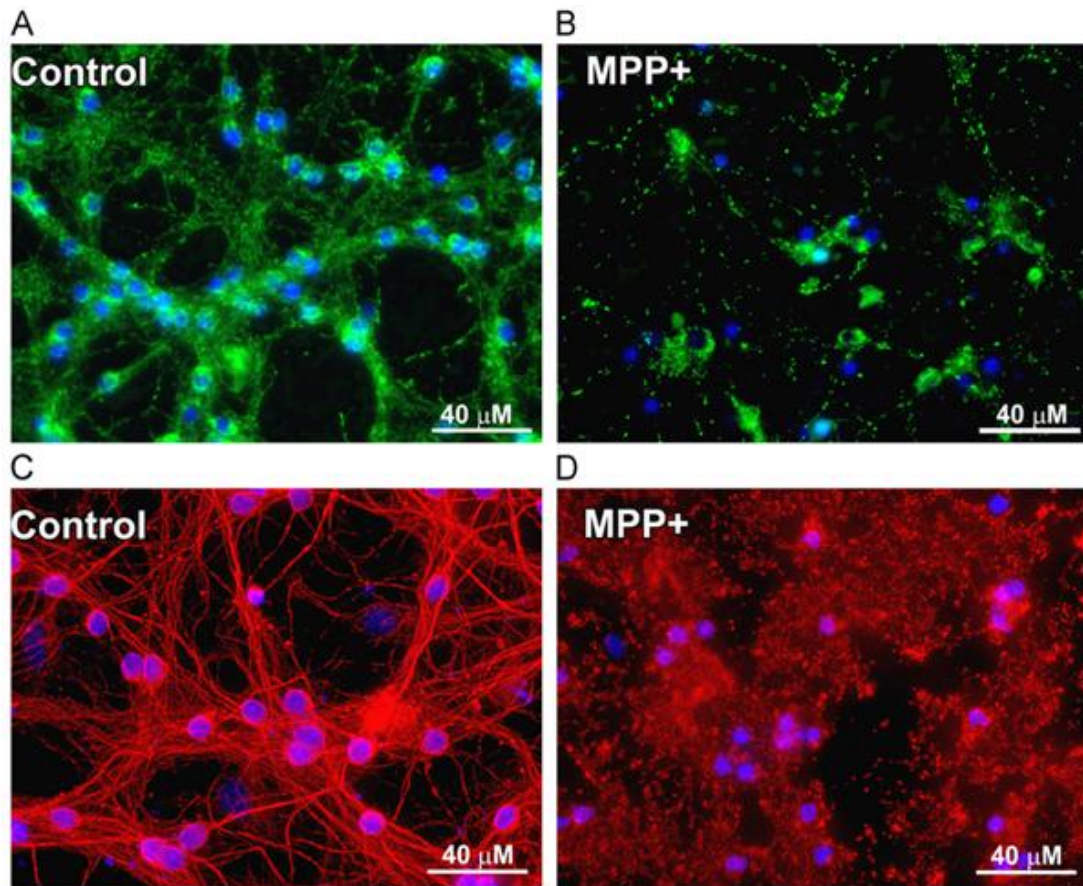


Fig. 2.4.4 - MPP⁺ induces striking mitochondrial fragmentation and severe microtubule disruption in CGNs. CGNs were incubated for 24 hours in either control medium (25K + serum) (A, C) or medium containing 150 μ M MPP⁺ (B, D). (A, B) Live imaging was performed using MitoTracker to detect mitochondria (green) and Hoechst to detect nuclei (blue). (C, D) CGNs were treated as in (A, B), fixed and immunostained for β -Tubulin (red). Nuclei were detected with DAPI (blue).

2.4.5 Rotenone-induced oxidative stress causes caspase-independent OPA1 cleavage, mitochondrial fragmentation, and microtubule disruption

To establish that the results seen with MPP⁺ treatment are a result of complex I inhibition and not a different aspect of its toxicity, CGNs were exposed to rotenone, another complex I inhibitor. Similar to results of MPP⁺ treatment, rotenone-treated cells also displayed cleavage of OPA1 to produce the fragment labeled “f”, which was not prevented by co-treatment with the pan-caspase inhibitor QVD (Fig. 5A). These data further support the notion that N-terminal cleavage of OPA1 induced by oxidative stress occurs in a *caspase-independent* manner. In addition, rotenone treatment rendered the mitochondrial network completely fragmented, demonstrated by scattered areas of small punctate foci detected by MitoTracker staining (Fig. 5B, C). Likewise, the microtubule network also displayed severe fragmentation comparable to that observed with MPP⁺ treatment (Fig. 5D, E).

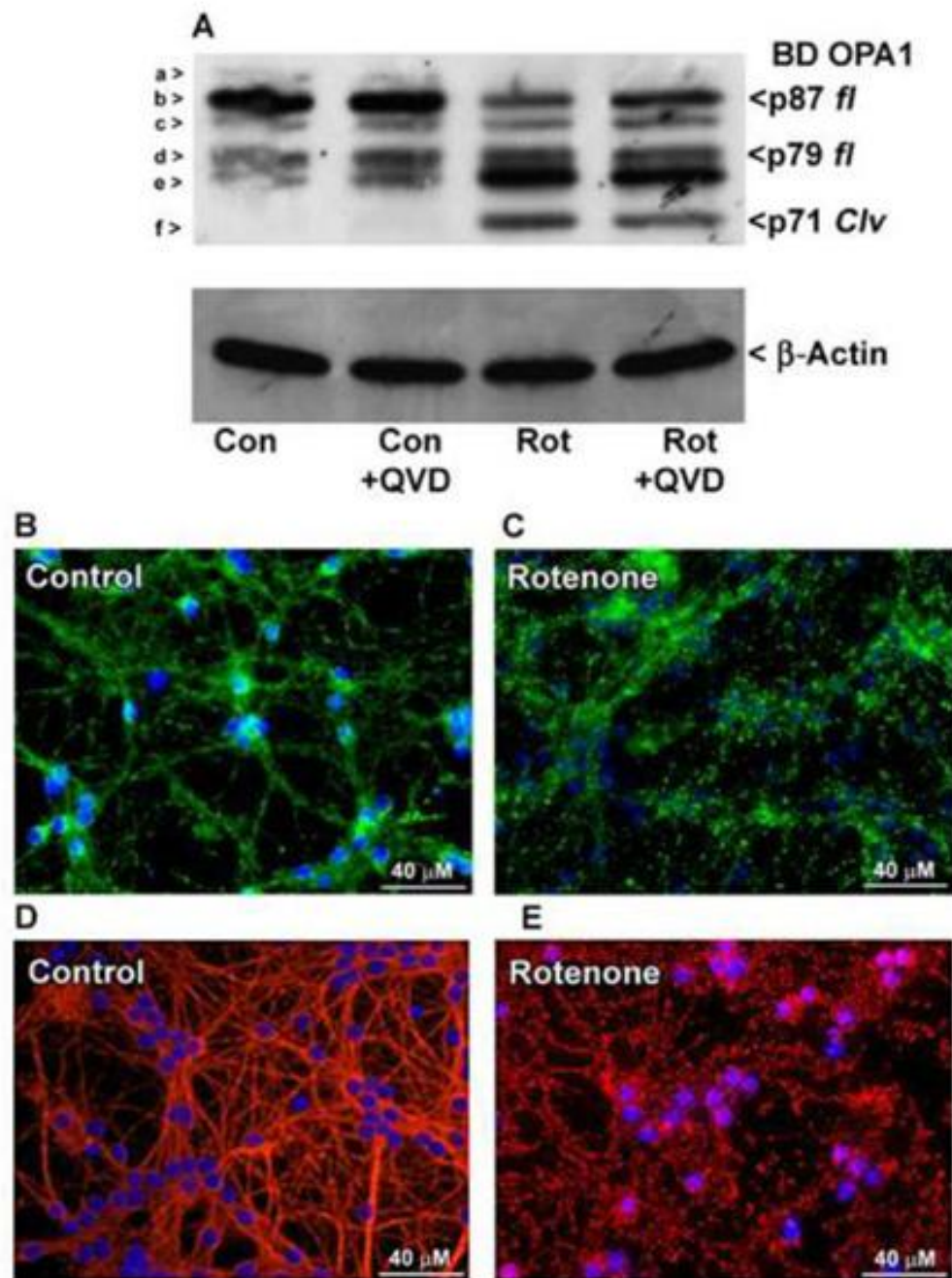


Fig. 2.4.5 - OPA1 cleavage induced by the complex I inhibitor rotenone is caspase-independent and occurs concurrently with mitochondrial and β -tubulin fragmentation. (A) CGNs were incubated for 24 h in either control medium (25K+serum) or medium containing ETC complex I inhibitor rotenone (Rot; 10 μ M) \pm broad spectrum caspase inhibitor QVD (10 μ M). OPA1 cleavage was detected by Western blotting with a monoclonal antibody to residues 708–830 (BD OPA1). The blot was then stripped and reprobed for β -Actin as a loading control. OPA1 isoforms a–f are indicated to the left in (A). p87 *fl* and p79 *fl* indicate full length OPA1 isoforms. p71 *clv* denotes the cleavage product. (B–E) CGNs were incubated for 24 h in either control medium (25K+serum) or medium containing 10 μ M rotenone. (B–C) Live imaging was performed using MitoTracker to detect mitochondria (green) and Hoechst to detect nuclei (blue). (D–E) CGNs were treated as in (B–C), fixed, and immunostained for β -Tubulin (red). Nuclei were detected with DAPI (blue).

2.4.6 RNS induces caspase-independent N-terminal cleavage of OPA1, mitochondrial fragmentation, and microtubule disassembly

Due to its high relevance to neurodegeneration, we examined the effects of RNS on OPA1 cleavage. CGNs were exposed to an overnight treatment with sodium nitroprusside (SNP), a nitric oxide donor. As expected, SNP induced N-terminal OPA1 cleavage that was resistant to co-treatment with the pan-caspase inhibitor QVD (Fig. 6A). SNP treatment also caused mitochondria to fragment in a manner similar to treatment with complex I inhibitors (Fig. 6B, C). In addition, SNP treatment caused disassembly and aggregation of the microtubule network (Fig. 6D, E). Again, this effect was more severe than what was observed with 5K treatment. These results indicate that RNS cause *caspase-independent* OPA1 cleavage and associated mitochondrial and microtubule fragmentation, similar to what is observed in the presence of ROS generated by inhibition of complex I.

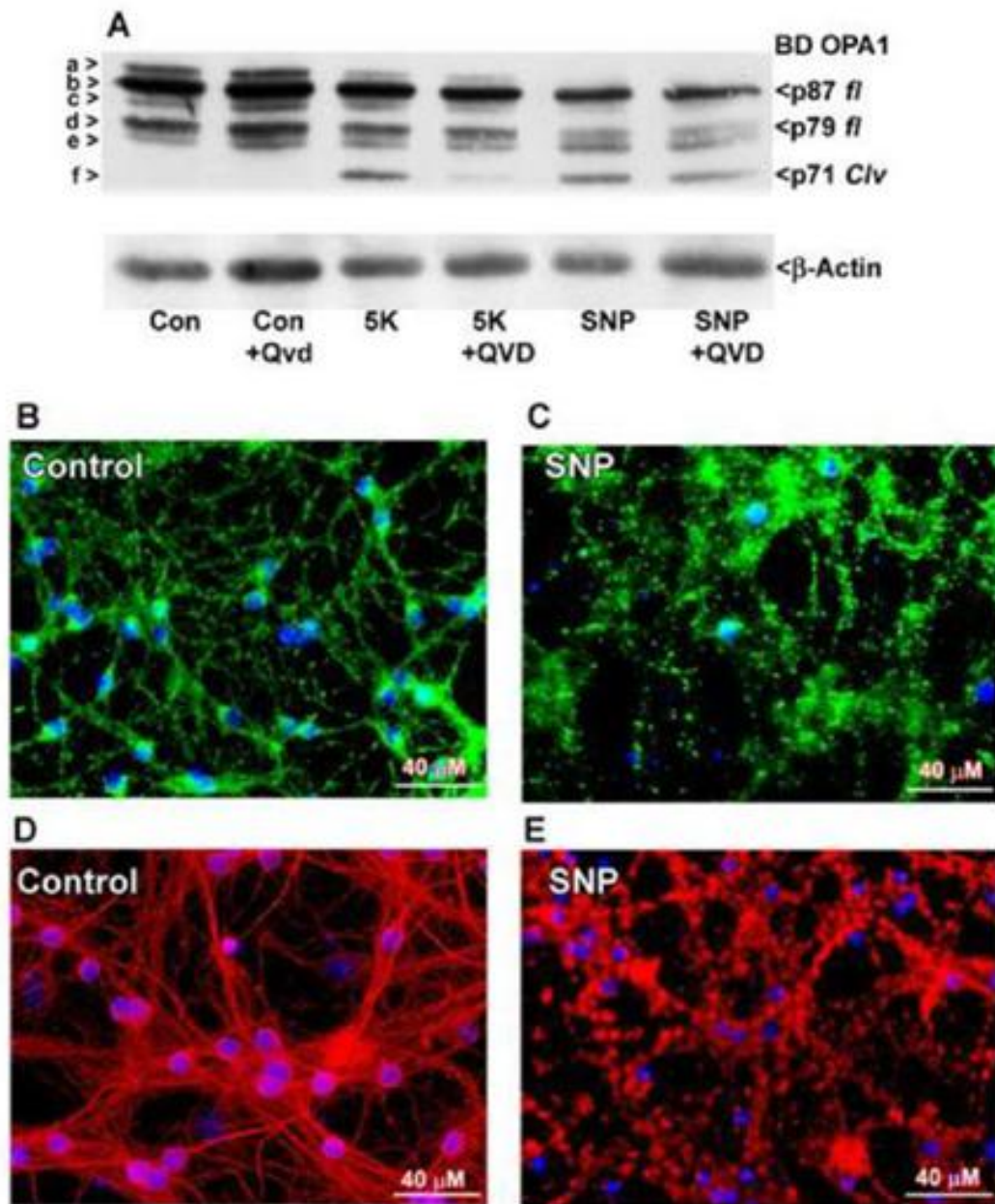


Fig. 2.4.6- OPA1 cleavage induced by the nitric oxide donor, SNP, is caspase-independent and occurs concurrently with mitochondrial fragmentation and microtubule disassembly. (A) CGNs were incubated for 24 h in either control medium (25K+serum) or medium containing the nitric oxide donor sodium nitroprusside (SNP) (75 μ M) +/- broad spectrum caspase inhibitor QVD (10 μ M). OPA1 cleavage was detected by Western blotting using a monoclonal antibody to residues 708–830 (BD OPA1). The blot was then stripped and reprobed for β -Actin as a loading control. OPA1 isoforms a–f are indicated to the left in (A). p87 *fl* and p79 *fl* indicate full length OPA1 isoforms. p71 *clv* denotes the cleavage product. (B–E) CGNs were incubated in either control medium (B, D) or medium containing 200 μ M SNP (C, E). (B, C) Live imaging was performed using MitoTracker to detect mitochondria (green) and Hoechst to detect nuclei (blue). (D–E) CGNs were treated as in (B–C), fixed, and immunostained for β -Tubulin (red). Nuclei were detected with DAPI (blue).

2.4.7 OPA1 cleavage induced by rotenone is resistant to inhibitors of JNK and p38 MAP kinases

It is well established that the JNK and p38 MAP kinase pathways are major contributors to cell stress responses during oxidative stress-induced apoptosis (Davis, 2000). More specifically, it has been demonstrated that the JNK and p38 MAP kinase pathways are significantly involved in rotenone-induced cell death (Newhouse et al., 2004). For this reason, we sought to examine the effects of inhibiting these pathways under conditions in which N-terminal cleavage of OPA1 occurs. CGNs were exposed overnight to rotenone or 5K treatments, with and without a JNK inhibitor (SP600125) or a p38 MAP kinase inhibitor (SB239063). Interestingly, these inhibitors attenuated 5K-induced OPA1 cleavage, although not to the same extent as the pan-caspase inhibitors BOC and QVD. This effect is likely due to the capacity of JNK and p38 MAP kinase inhibitors to decrease CGN apoptosis in response to 5K treatment and as a result, diminish caspase activity. Surprisingly, rotenone-induced OPA1 cleavage was completely insensitive to JNK and p38 MAP kinase inhibition (Fig. 2.4.7). These data further support a distinct pathway for oxidative stress-induced OPA1 cleavage from that of classically apoptotic conditions.

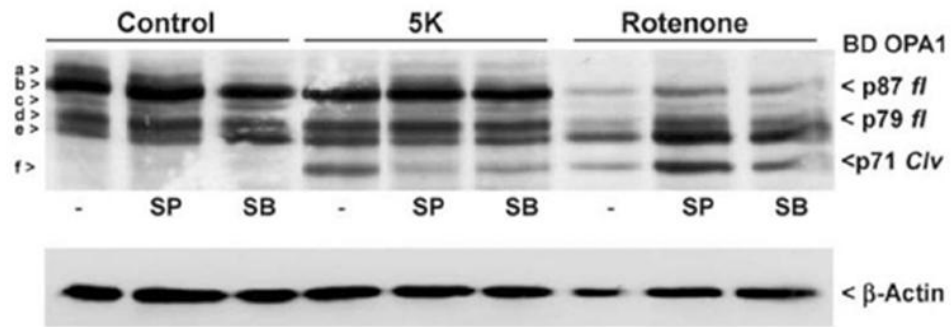


Fig. 2.4.7 - OPA1 cleavage induced by 5K treatment or rotenone demonstrate differential resistance to inhibition of JNK and p38 MAP kinase. CGNs were co-incubated for 24 h in either control medium (25K+serum), 5K medium, or medium containing the complex I inhibitor rotenone (10 μ M) +/- the JNK inhibitor II SP600125 (10 μ M) or the p38 MAP kinase inhibitor SB239063 (10 μ M). OPA1 cleavage was detected by Western blotting using a monoclonal antibody to residues 708–830 (BD OPA1). The blot was stripped and reprobed for β -Actin as a loading control. OPA1 isoforms a–f are indicated to the left. p87 *fl* and p79 *fl* indicate full length OPA1 isoforms. p71 *clv* denotes the cleavage product.

2.4.8 N-terminal cleavage of OPA1 occurs in aged rat and mouse brain tissues

Increased oxidative damage has been demonstrated to be an attribute of the aging process, particularly in the brain. In the preceding results, we consistently demonstrate OPA1 cleavage in the presence of various oxidative or nitrosative stressors. Therefore, it follows that the increased oxidative stress characteristic of aged brain tissue may similarly lead to OPA1 cleavage. To examine this hypothesis, we obtained midbrain and hippocampal tissue from young (4 month-old) and aged (22 month-old) rats in order to compare processing of endogenous OPA1. Pooled (from 3 rats) midbrain and hippocampal tissues from aged rats displayed an OPA1 cleavage product identical in molecular weight to that observed under oxidative stress and apoptotic conditions *in vitro* (Fig. 8A). This cleavage product was absent in other aged brain tissues, such as the cerebellum and substantia nigra (data not shown). We next examined endogenous OPA1 cleavage in young (4 month-old) and old (15 month-old) tissues from individual mice. Significant endogenous OPA1 cleavage was observed in hippocampus and midbrain but not cerebellum and cortex. Of note, there was a considerable increase in endogenous OPA1 cleavage observed in aged mouse hippocampal tissue, as compared to other brain tissues examined (Fig. 8B, C). These results are consistent with increased oxidative stress in aged brain tissue and illustrate that OPA1 cleavage may provide a mechanistic link between mitochondrial dysfunction, oxidative stress, and neurodegeneration associated with aging.

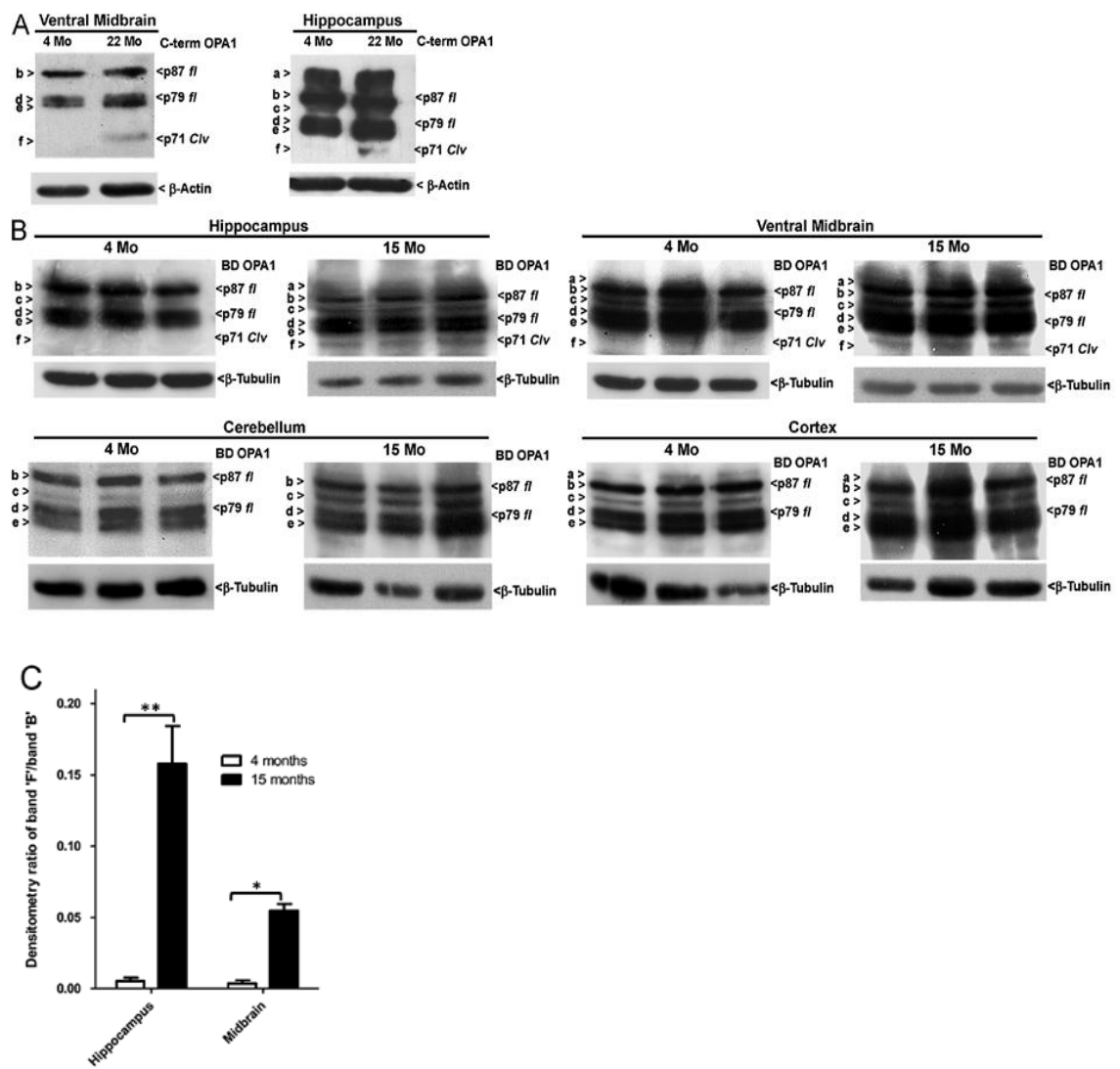


Fig. 2.4.8 - OPA1 cleavage is observed in aged midbrain and hippocampal tissues.

(A) Midbrain and hippocampal tissues were obtained from young (4 months) or aged (22 months old) rats, homogenized, and Western-blotted for spontaneous OPA1 cleavage, without exposure to exogenous insults. OPA1 was detected by Western blotting with a polyclonal antibody to the C-terminal 14 amino acids, residues 947–960 (C-term). Blots were then stripped and reprobed for β -Actin as a loading control. (B) Hippocampal, midbrain, cerebellum, and cortical tissues were obtained from young (4 months old) or aged (15 months old) mice, homogenized as in (A), and Western-blotted for spontaneous OPA1 cleavage, without exposure to exogenous insults. OPA1 cleavage was detected by Western blotting using a monoclonal antibody to residues 708–830 (BD OPA1). Blots were then stripped and reprobed for β -Tubulin as a loading control. OPA1 isoforms a–f are indicated to the left. p87 *fl* and p79 *fl* indicate full length OPA1 isoforms. p71 *clv* denotes the cleavage product. (C) Relative optical density of band f observed in young and aged mouse tissue was obtained for each mouse by calculating the ratio of the density of band “f” to band “b”. Results were graphed as mean \pm SEM of each of the three animals for each age and tissue type tested. * $=p<0.05$, ** $=p<0.01$, $n=3$.

2.5 Discussion

Mitochondrial fission and fusion in mammalian cells are controlled by the dynamin family GTPases DRP1, OPA1, and the mitofusins, MFN1 and MFN2. The OPA1 GTPase has two main functions within mitochondria. First, OPA1 works in concert with its binding partner, mitofusin, to promote mitochondrial fusion (Cipolat et al., 2004). Second, long and short forms of OPA1 combine within oligomer complexes that maintain tight cristae junctions (Frezza et al., 2006). OPA1 plays a critical role in each of these functions as demonstrated by the significant mitochondrial fragmentation and disruption of the cristae architecture induced by knockdown of OPA1 expression (Olichon et al., 2003; Griparic et al., 2004). Imbalances in the fission and fusion equilibrium of mitochondria have been linked mechanistically to apoptotic cell death pathways. For instance, the pro-apoptotic Bcl-2 family member, Bax, has been shown to localize to mitochondrial fission sites and, in the case of nitric oxide-induced apoptosis in cortical neurons, requires DRP1 function in order to accumulate in foci along the mitochondrial outer membrane (Karbowski et al., 2002; Yuan et al., 2007). Thus, under some conditions enhanced mitochondrial fission appears to be required upstream of mitochondrial outer membrane permeabilization and cytochrome *c* release. On the other hand, Bcl-2 homology-3 domain (BH3)-only proteins including Bid and Bnip3, disrupt OPA1 oligomer formation and act in concert with Bax to induce cristae remodeling and promote a maximal release of cytochrome *c* during induction of apoptosis (Frezza et al., 2006; Yamaguchi et al., 2008; Landes et al., 2010a; Landes et al., 2010b). Collectively, these studies suggest that loss of OPA1 function predisposes cells to apoptosis via two

independent pathways including decreased mitochondrial fusion and loosening of cristae junctions. Therefore, unraveling the regulation of OPA1 function is essential to mechanistically define the link between disruption of mitochondrial fission/fusion and induction of apoptosis.

Proper mitochondrial location within the neuron is mediated via microtubule transport. Thus, impairment of this critical network has a direct effect on mitochondrial physiology (Saxton and Hollenbeck, 2012). Our data reveal concomitant fragmentation of the microtubule and mitochondrial networks during oxidative stress. This is in accord with previous literature reporting that peroxynitrite exposure impairs microtubule formation (Landino et al., 2002). More specifically, peroxynitrite and hydrogen peroxide treatments result in cysteine oxidation of microtubule-associated-protein 2 (MAP2) and tau, inhibiting their ability to promote microtubule assembly (Landino et al., 2004). Complex I inhibition by MPP⁺ and rotenone have been shown to interfere with microtubule assembly through direct binding and destabilizing of tubulin (Marshall and Himes, 1978; Cappalletti et al., 2005). Through the establishment that oxidative stress insults have direct effects on the microtubule network, it can be inferred that our observations of microtubule fragmentation are likely from a similar mechanism. The effects of microtubule fragmentation is also linked to perpetuation of the apoptotic cascade, through sequestration of pro-apoptotic BH3-only proteins, such as Bim and Bmf, thereby limiting their ability to bind pro-survival Bcl-2 proteins (Day et al., 2004). This is consistent with reports of apoptotic stimuli disrupting the interaction of the cytoplasmic dynein light chain and the potent pro-death Bim, allowing for inhibition of

pro-survival Bcl-2 (Puthalakath et al., 1999). Therefore, it is likely that the microtubule fragmentation observed in our studies results in a similar perpetuation of apoptotic signaling. On the other hand, decreased ATP synthesis resulting from breakdown of mitochondrial function will impair the structure of the tubulin network, thereby propagating microtubule associated apoptotic signaling (Oropesa et al., 2011). Hence, the probability exists that the damage to mitochondria and microtubules observed in our studies has a cyclical property which leads to further breakdown of these critical networks.

OPA1 function is largely regulated through post-translational proteolytic processing within the mitochondria. A relatively large number of mitochondrial proteases including PARL, YME1L1, AFG3L1 -2, Paraplegin, and OMA1, have all been shown to cleave OPA1 under certain conditions (Cipolat et al., 2006; Ishihara et al., 2006; Griparic et al., 2007; Song et al., 2007; Guillery et al., 2008; Ehses et al., 2009). These cleavage events typically result in the processing of long isoforms of OPA1 (denoted “a” and “b” on Western blots) into short isoforms (denoted “c”, “d”, and “e” on Western blots). These long and short isoforms then interact to form functional OPA1 oligomers that promote mitochondrial fusion and cristae architectural integrity (Landes et al., 2010a; Landes et al., 2010b). In fact, a non-cleavable long form of OPA1 is unable to induce mitochondrial fusion when expressed in OPA1 null cells; however, co-expression of short forms of OPA1 restores this fusion capacity (Song et al., 2007). On the other hand, there is also ample evidence that aberrant cleavage of OPA1 which produces an overabundance of short forms disrupts the mitochondrial fusion machinery leading to fragmentation; a

situation that occurs during pathological conditions such as loss of mitochondrial membrane potential or induction of apoptosis (Duvezin-Caubet et al., 2006; Ishihara et al., 2006).

Specifically within the context of OPA1 processing during apoptosis, we have previously identified a novel N-terminal cleavage of OPA1 that occurs during apoptotic conditions in primary cultured CGNs and human neuroblastoma SH-SY5Y cells. This cleavage event results in the formation of an N-terminal truncated “short” form of OPA1 (denoted “f” on Western blots), that is significantly smaller than a deletion mutant of OPA1 lacking the N-terminal 300 amino acids (Loucks et al., 2009). Thus, this novel short form of OPA1 observed during neuronal apoptosis lacks the critical residue, K301, which is essential for the GTPase function of OPA1 (Griparic and van der Bliek, 2005). Although this cleavage event was determined to be caspase-dependent, caspases failed to directly cleave OPA1 *in vitro*. In the current study, we reveal an identical OPA1 cleavage event that occurs during oxidative and nitrosative stress-induced cell death in CGNs. The observed OPA1 cleavage product is indistinguishable from that previously observed during classically apoptotic conditions (5K) in CGNs, based on size comparison and recognition with both C-terminal specific antibodies and antibodies specific to the middle portion of OPA1. However, in contrast to our previous findings, co-incubation with the pan-caspase inhibitors, BOC and QVD, failed to inhibit the cleavage of OPA1 induced by oxidative or nitrosative stress conditions in CGNs. This result indicates that OPA1 cleavage occurs in a caspase-*independent* manner under these conditions, as opposed to the caspase-*dependent* mechanism observed during 5K-induced CGN apoptosis. Notably,

we also demonstrate that this OPA1 cleavage product is present in midbrain and hippocampal tissues obtained from aged mice and rats (15-month-old, 22-month-old, respectively), whereas no OPA1 cleavage product was observed in corresponding tissues taken from young (4-month-old) mice or rats. This finding suggests that OPA1 cleavage may provide a point of convergence between oxidative stress, mitochondrial dysfunction, and aging; a mechanistic link that may be particularly relevant to the enhanced risk of neurodegeneration associated with advancing age.

To date, despite testing a large number of protease inhibitors, we have been unable to identify the protease that cleaves OPA1 to generate the novel cleavage product ("F") described above. Based on our previous and current findings, we postulate that two distinct mechanisms converge to activate the mitochondrial protease responsible for the N-terminal cleavage of OPA1. The first mechanism involves regulation of the unknown OPA1 protease by a caspase. Caspase-mediated activation of proteins is well established in the apoptotic pathway, such as the activation of executioner caspases by initiator caspases or the caspase-8 mediated activation of the pro-apoptotic BH3-only protein Bid (Li et al., 1998). In addition, caspases have also been shown to remove the regulatory domains of a large number of protein kinases, often resulting in heightened or constitutive activation of the kinase (Lee et al., 1997; Takahashi et al., 1998; Chen et al., 1999; Basu et al., 2002; Huang et al., 2002). Therefore, it is reasonable to hypothesize that in the case of caspase-dependent OPA1 cleavage, activated caspases may directly cleave a negative regulatory domain of the unidentified OPA1 protease resulting in its activation. This is consistent with our observations that caspases are necessary for

apoptosis-induced N-terminal cleavage of OPA1, but are incapable of directly cleaving OPA1 *in vitro* (Loucks et al., 2009). The OPA1 cleavage observed under conditions of oxidative or nitrosative stress apparently involves activation of the unidentified OPA1 protease through a caspase-independent mechanism. One possibility is that this protease could be activated by an oxidative or nitrosative stress-dependent phosphorylation event. However, this seems unlikely since OPA1 cleavage induced by oxidative stress was not significantly affected by inhibitors of JNK or p38 MAP kinases. Another possibility is that the unidentified OPA1 protease is normally sequestered and maintained in an inactive state by its association with the mitochondrial lipid cardiolipin. During conditions of oxidative or nitrosative stress, this protease may then become activated by the oxidation of cardiolipin, perhaps in a manner similar to cardiolipin oxidation allowing for mobilization of cytochrome *c* (Shidoji et al., 1999). In this context, it is interesting to note that anthocyanins, polyphenolic antioxidants found in fruits and vegetables, significantly inhibit both cardiolipin oxidation and OPA1 cleavage in CGNs exposed to a Bcl-2 inhibitor (Kelsey et al., 2011). Finally, a protease may undergo a direct conformational change as a result of oxidation or nitrosylation, resulting in its activation. Thus, there are numerous possible mechanisms by which a mitochondrial protease might become activated to cleave OPA1 under oxidative or nitrosative stress conditions. Further studies, including identification of the precise cleavage site, are necessary in order to narrow down the search for the mitochondrial protease involved in generating the novel short form (“f”) from OPA1.

The relevance of the present findings to neurodegeneration is supported by the extensive literature demonstrating a significant impact of pathogenic proteins on the mitochondrial fission and fusion machinery in various disease states. For instance, alterations in the expression of multiple fission and fusion proteins have been reported in AD brain, including a significant reduction in OPA1 (Wang et al., 2009). Moreover, oligomeric amyloid-beta-derived diffusible ligands or overproduction of amyloid beta via overexpression of Swedish mutant amyloid precursor protein (APP^{swe}), have each been shown to induce mitochondrial fragmentation in cultured neuronal cells (Wang et al., 2008; Wang et al., 2009). In the case of neuronal cells overexpressing APP^{swe}, simultaneous OPA1 overexpression rescues the mitochondria from fragmentation. In a similar manner, the PD genes, pink1 and parkin, have been shown to negatively regulate OPA1 function and induce mitochondrial fission in *Drosophila* (Deng et al., 2008). In accordance with these findings, RNAi-induced knockdown of Pink1 in rat hippocampal or midbrain dopaminergic neurons induces hyperfusion of mitochondria, an effect that is suppressed by concurrent knockdown of OPA1 (Yu et al., 2011). Finally, mutant Huntingtin protein has recently been shown to bind to DRP1 and enhance its GTPase activity, resulting in significant mitochondrial fragmentation in neurons *in vitro* and *in vivo* (Song et al., 2011). Collectively, these studies suggest that aberrant activation or suppression of the mitochondrial fission or fusion machinery likely plays a significant pathogenic role in a number of neurodegenerative disorders. Given these findings, it will be interesting to determine if aberrant processing of OPA1, particularly the generation of

the N-terminal truncated cleavage product described here, occurs in disorders such as AD, PD, and Huntington's disease.

Loss of function of OPA1 has also been linked to mitochondrial dysfunction, mitochondrial oxidative stress, and impaired mitochondrial metabolism. For example, mutations in OPA1 which are causative in dominant optic atrophy cause reductions in ATP synthesis in both fibroblasts and skeletal muscle (Zanna et al., 2008; Lodi et al., 2011). Furthermore, a subset of patients who exhibit mutations in the highly conserved GTPase domain of OPA1 demonstrate multiple phenotypes in addition to progressive blindness, such as deafness, ataxia, and neuropathy. These patients also exhibit increased mutations in mitochondrial DNA, a condition known to be a hallmark of aging (Amati-Bonneau et al., 2008). In accordance with these findings in patients, mice displaying haploinsufficiency of the OPA1 protein demonstrate pathological features of dominant optic atrophy affecting the retinal ganglion cells, but show more subtle neuromuscular and metabolic abnormalities as well (Alavi et al., 2007; Alavi et al., 2009). Moreover, these mice also display upregulation of NMDA receptors and enhanced oxidative stress in the retina (Nguyen et al., 2011). This link between OPA1 loss of function and mitochondrial dysfunction and oxidative stress also appears to be significantly associated with aging. For instance, OPA1 expression is lower in skeletal muscle of elderly humans compared to young controls (Joseph et al., 2012). Additional studies suggest that this observation may be more than just correlative since heterozygous mutation of OPA1 in *Drosophila* or deletion of the OPA1 ortholog, Mgm1p, in the yeast *S. cerevisiae*, induces a significant shortening of lifespan in each of these model systems (Tang et al., 2009;

Scheckhuber et al., 2011). Given the mechanistic link between OPA1 loss of function, mitochondrial dysfunction, oxidative stress, and aging, it is not surprising that we observed OPA1 cleavage in aged rat and mouse midbrain and hippocampal tissues. OPA1 cleavage to generate the novel short form “f” may in fact be a possible biomarker of aging and the declining mitochondrial function associated with this process.

In summary, we propose a model in which an unidentified mitochondrial protease becomes activated as a result of ROS/RNS, increasing age, or through activation of caspases as a result of induction of apoptosis. This mitochondrial protease cleaves the N-terminus of OPA1 removing the key residue K301, and rendering the novel short form of OPA1 (“f”) GTPase deficient. This cleavage event results in a loss of OPA1-dependent fusion, leading to enhanced mitochondrial fission and dismantling of cristae junctions which likely contribute to further mitochondrial dysfunction, oxidative stress, and induction of apoptosis. We hypothesize that these mechanisms become more pronounced with increasing age as a result of increased ROS/RNS production. Our data suggest that aberrant OPA1 cleavage may be a point of convergence that mechanistically links impairment of the mitochondrial fission/fusion equilibrium with mitochondrial dysfunction, mitochondrial oxidative stress, and neurodegenerative disorders associated with aging (Fig. 2.4.9). Future studies aimed at identifying the mitochondrial protease responsible for the OPA1 cleavage event described here may reveal a novel molecular player in aging and neurodegeneration that could be targeted therapeutically to maintain OPA1 function and limit mitochondrial dysfunction in the CNS.

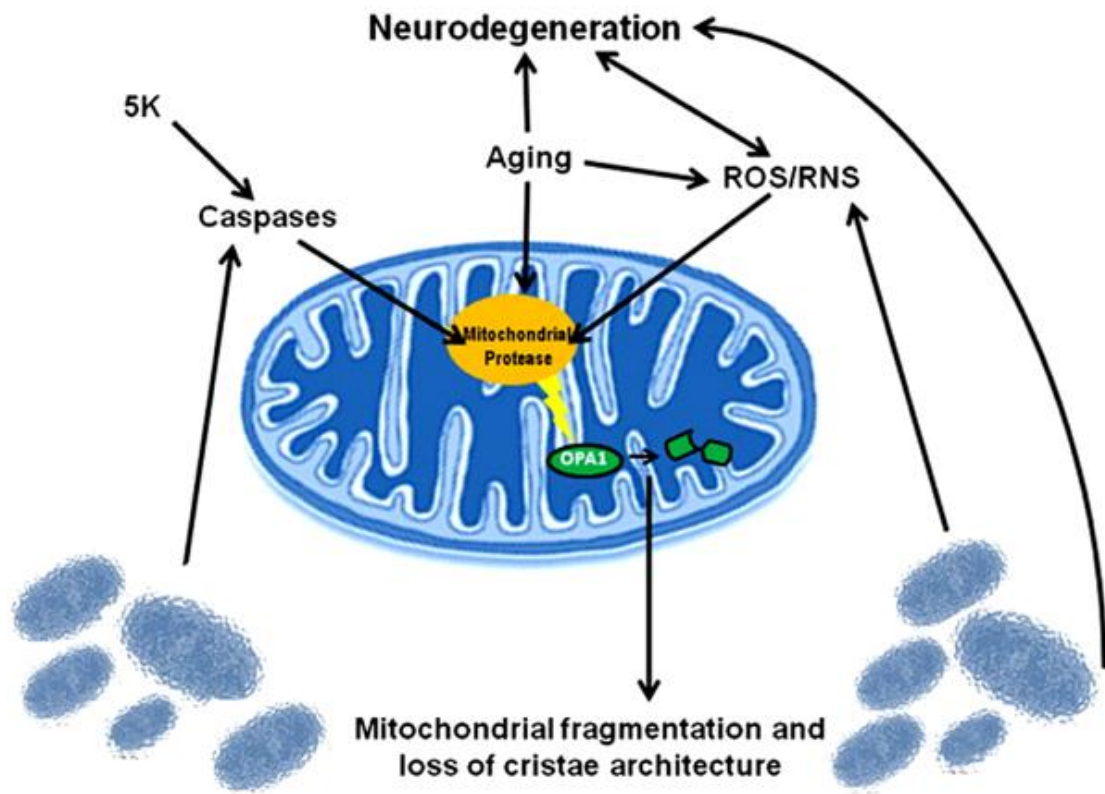


Fig. 2.4.9 - Schematic of proposed OPA1 cleavage mechanism. We propose a mechanism in which an unknown protease can become activated through two different pathways. One pathway involves a caspase-dependent activation, as observed under classically apoptotic conditions (5K). A secondary pathway involves activation of the unknown protease in a caspase-independent manner resulting from ROS/RNS stress. Upon activation of the protease, OPA1 becomes cleaved, producing a GTPase-deficient, non-functional C-terminal fragment. Impairment of proper OPA1 GTPase function results in loss of cristae architecture, mitochondrial fragmentation, and ultimately cell death. The ensuing breakdown of mitochondrial function causes further increases in ROS, resulting in continued cleavage of OPA1 and perpetuation of this cell death cascade. Our data demonstrates that OPA1 cleavage occurs spontaneously in aged brain tissue, likely resulting from increased oxidative stress that occurs during the aging process. OPA1 cleavage in aged brain tissue likely contributes to the cell death cascade observed during aging and in neurodegeneration.

Chapter Three: TAR-DNA binding protein-43 (TDP-43) localization to mitochondria suggests a novel role in modulating mitochondrial physiology

3.1 Abstract

Protein aggregation is a common pathogenic mechanism in diverse neurodegenerative disorders. TAR DNA-binding protein-43 (TDP-43) cytoplasmic inclusions are pathological hallmarks of several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Furthermore, TDP-43 mutations have been identified in both familial and sporadic forms of ALS. Despite evidence linking dysfunction of TDP-43 to neuronal cell death, it is currently unknown whether TDP-43-induced toxicity occurs via a gain of function or loss of function mechanism. In the present study, we initially examined the effects of either overexpression or knockdown of TDP-43 on neuronal survival *in vitro*. Overt neuronal cell death was not induced by either experimental paradigm. However, it was noted that both endogenous and overexpressed TDP-43 partially localized to mitochondria within diverse neuronal and astrocytic cell types. Furthermore, a significant decrease in the function of mitochondrial ETC Complex I occurred following TDP-43 knockdown in NSC34 motor neuron/neuroblastoma hybrid cells. Our data suggest a novel link between TDP-43 localization and function and mitochondrial physiology.

3.2 Introduction

TDP-43 is an hnRNP protein that was first discovered because of its ability to bind the transactive response region of HIV-1, where it represses gene expression (Ou et al., 1995). Since its discovery, TDP-43 has been consistently demonstrated to be involved at various levels of gene expression, such as modulation of exon splicing (Buratti et al., 2001; Mercado et al., 2005; Ayala et al., 2008), modulation of gene expression through direct DNA and RNA binding (Ayala et al., 2005; Acharya and Reddi, 2006; Strong et al., 2007), and modulation of gene expression through stabilization of mRNA (reviewed in Chen-Plotkin et al., 2010). In addition to direct interactions with RNA and DNA, TDP-43 also participates in protein-protein interactions through its C-terminal glycine-rich domain, notably with other hnRNP proteins involved in alternative splicing (Buratti et al., 2005; D'Ambrogio et al., 2009). Although predominantly located in the nucleus, TDP-43 can be exported via its nuclear export signal (NES) to perform functions in the cytoplasm (Ayala et al., 2008; Winton et al., 2008).

Perhaps the most notable recent finding in regards to ALS pathology is that cytoplasmic ubiquitin-positive inclusions containing TDP-43 are consistently found in *both* sporadic and most familial forms of the disease (Arai et al., 2006; Neumann et al., 2006; Davidson et al., 2007; Dickson et al., 2007; Brandmeir et al., 2008). The significance of this discovery is underscored in that other histopathological findings in familial and sporadic forms of ALS are seemingly heterogeneous. As such, evaluation of

the mechanism behind dysregulation and aggregation of TDP-43 has become a recent focus in ALS research.

In addition to TDP-43 pathological findings, mutations in TDP-43 are also found in a subset of familial ALS cases. The Ala-315-Thr (A315T) mutation in TDP-43 was initially discovered in a genetic screen of families exhibiting an autosomal dominant inheritance of ALS (Glitcho et al., 2008). This residue is highly conserved within the glycine-rich domain, indicative of its functional importance. ALS patients harboring the A315T TDP-43 mutation are affected by significant TDP-43 histopathology concomitant with extensive motor neuron loss in the spinal cord. Although it is accepted that mutations in TDP-43 are involved in some forms of ALS, the direct toxic effects of TDP-43 mutations to neurons remain to be established.

Despite the preponderance of evidence demonstrating that TDP-43 inclusions are an almost universal finding in the pathophysiology of ALS and FTLN, the processes behind TDP-43 mediated toxicity remain to be fully elucidated. Studies into the effects and mechanisms of TDP-43 aggregation have produced varying results, yet the one factor that remains consistent is that increased TDP-43 cytoplasmic localization is commensurate with a marked loss of nuclear TDP-43. A central challenge in understanding how TDP-43 defects mediate neuronal cell death is ascertaining whether toxicity occurs from gain of function (GOF) effects due to aberrant cytoplasmic localization or loss of function (LOF) effects due to a deficiency in nuclear TDP-43 functions. Indeed, current literature supports both options, and a dichotomy remains on the answer to this question.

In the present study, we used the NSC-34 motor neuron/neuroblastoma hybrid cell line to evaluate the effects of overexpressing GFP-tagged wild-type (WT) or A315T mutant TDP-43 in this system. Additionally, we used siRNA to effectively knock-down expression of endogenous TDP-43. Consistent with previous findings, we noted that exogenous GFP-TDP-43 had a predominantly nuclear localization along with noticeable cytoplasmic staining, using both WT and A315T constructs. However, neither overexpression of WT or A315T TDP-43 or knock-down of endogenous TDP-43 had overt toxic effects to NSC-34 cells. We were able to demonstrate that GFP-tagged WT and A315T TDP-43, as well as endogenous TDP-43, localized to the mitochondria in various cell types. Furthermore, depletion of endogenous TDP-43 caused a significant decrease in mitochondrial Complex I activity. Collectively, these findings indicate a potential novel function of TDP-43 in facilitating mitochondrial function and physiology.

3.3 Materials and Methods

3.3.1 Materials

The monoclonal antibody to TDP-43 was obtained from ProteinTech (Chicago, IL, USA). The monoclonal antibody to OPA1 (residues 708–830) was obtained from BD Biosciences (San Diego, CA, USA). Ethacrynic acid and primary antibody against β -tubulin, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay, Geneticin (G418) and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (Anti-GAPDH) was from Abcam (Cambridge, MA, USA). Horseradish peroxidase-linked secondary antibodies and

reagents for enhanced chemiluminescence were from GE Healthcare (Pittsburgh, PA, USA). The monoclonal β -tubulin antibody was from Sigma-Aldrich (St. Louis, MO, USA). A CY3-conjugated secondary antibody for immunofluorescence was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). DharmaFECT Transfection Kit, smart pool siRNA against mouse TDP-43, and non-target siRNA were from Thermoscientific Pierce Biotechnology (Rockford, IL, USA). Optimem and Dulbecco's modified Eagle's medium were purchased from Gibco (Carlsbad, CA, USA). NSC34 cells were kindly provided by Dr. Neil Cashman (University of British Columbia). GFP-tagged wild type and A315T mutant TDP-43 constructs were kindly provided by Dr. Jane Wu at Northwestern University.

3.3.2 Cell Culture

NSC-34 cells were plated at a concentration of 250,000 cells per well in a tissue culture treated 6-well culture vessel and maintained in high-glucose DMEM, 10% FBS, 1% pen-strep. Subsequent to initiation of the siRNA protocol, the media was changed to antibiotic free media per manufacturer's recommendations. In general, treatments were initiated 48 hours after plating and transfections were initiated 24 hours after plating.

3.3.3 Transfection procedure

Transfections were carried out in NSC-34 cells 24 hours post-plating following standard Lipofectamine 2000 protocol. Cells were transfected with 5 μ g GFP-tagged wild type TDP-43, A315T TDP-43 or GFP control vectors as indicated in the results section. In general, expression was carried out for 48 or 72 hours, as indicated in the results section.

3.3.4 Cell lysis and immunoblotting

After treatment as described in the Results section, cells were washed once in 1 mL ice-cold phosphate-buffered saline (PBS) (pH 7.4). Cells were incubated for 10 min on ice in lysis buffer (150–200 µl per 35-mm well) prepared as described previously (Loucks et al., 2006). The cells were then harvested by scraping, and cell debris were removed by centrifugation at 6000 g for 2 min. Protein concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) with final absorbance of samples measured by a microplate spectrophotometer (BioTek PowerWave XS2, Winooski, VT, USA). Whole cell lysates were subjected to SDS-PAGE through 7.5% polyacrylamide gels. Resolved proteins were transferred to PVDF membranes and processed for immunoblot analysis as previously described (Loucks et al., 2006). In general, western blots shown are representative of a minimum of three independent experiments.

3.3.5 Immunofluorescence

After treatment as described in the Results section, cells were fixed for 1 h at room temperature in 4% paraformaldehyde followed by permeabilization and blocking in 0.2% Triton X-100 and 5% BSA in 1X PBS (pH 7.4) for 1 hour. Cells were incubated in DAPI diluted in 2% BSA and 0.2% Triton X-100 in PBS for 30 minutes. Cells were then washed five times in 1X PBS followed by addition of anti-quench (5 mg/ml *p*-phenylenediamine in 1X PBS).

3.3.6 Cell Fractionation

Cells were treated as indicated in the results section. Media was aspirated, and cells were washed once with 1X ice-cold phosphate-buffered saline (PBS), pH 7.4. One ml of cytosolic buffer provided in the kit was diluted 1:5 in ddH₂O, with added protease inhibitor cocktail (supplied in kit), and 1 mM dithiothreitol (DTT) as per the manufacturer's recommendations. A 100-μL aliquot of the cytosolic buffer was added per well and incubated on ice for 20 min, at which point cells were harvested and homogenized with 60-80 passes of a Dounce pestle. Effective homogenization at this step was confirmed microscopically. A whole cell lysate sample was collected at this point and added to wahl lysis buffer. Samples were then spun down at 720 g for 10 min at 4°C two times, with the supernatant removed and placed in a fresh tube each time. The supernatant was transferred to a fresh tube and spun tube down at 10 000 g for 30 min at 4°C to collect the mitochondrial pellet, which was resuspended in 100 μL of mitochondrial buffer (provided in the kit) with protease inhibitor cocktail and 1 mM dithiothreitol as per the manufacturer's recommendations. The remaining cytosolic supernatant was transferred to a fresh tube. Samples were immediately used for further experiments or stored at -20 °C.

3.3.7 MTT viability assay

After treatment as indicated in the Results section, NSC-34 cells were incubated with 100 μL of 12 mM MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 4 h at 37°C, 10% CO₂. 2 ml of dimethyl sulfoxide

(DMSO) was then added to solubilize the formazan and the plate was read at 570 nm in a spectrophotometer.

3.3.8 SMARTPOOL[®] siRNA mediated knockdown of TDP-43

NSC-34 cells were transfected with either the SMARTPOOL[®] (Thermo Scientific, Pittsburgh, PA, USA) siRNA against mouse TDP-43 or the non-target siRNA pool using the DharmaFECT transfection protocol. Briefly, 50-100 nM of indicated siRNA per well was incubated with 5 µL of DharmaFECT transfection reagent separately with serum-free medium for 5 min at ~25°C. siRNA and DharmaFECT in serum-free medium were mixed and incubated at ~25°C for 20 min. The mixture of siRNA/DharmaFECT was then added to the cells and incubated for 24 h at 37°C and at 10% CO₂ before subsequent experiments were completed.

3.3.9 Oroboros

High resolution respirometry was carried out using Oxygraph 2-k, (O2k) Oroboros Instrument as described in Hughey et al. (2011) and Kane et al. (2011). Briefly, following 72 hour siRNA construct expression, cells were trypsinized, and cell counts were obtained. 250,000 cells in MiR05 were used per each 2 ml oxygraph chamber and resuspended in MiR05 media. Using a 10 µL Hamilton microsyringe, cells were permeabilized using titrations of 0.3 µL 10mM digitonin until baseline respiration was achieved. Using 25 µL Hamilton microsyringe, 5 mM pyruvate, 2 mM malate, and 10 mM glutamate (final concentrations) were injected into each system to establish leak state respiration. 2 µL injections of 0.5 M ADP were titrated until respiration began to decrease in control samples. 6 µL Succinate, 5 µL cytochrome *c*, 5 µL oligomycin, and 1

μL FCCP were titrated at 1 M, 4 mM, 4 mg/ml, and 1 mM stock concentrations, respectively.

3.4 Results

3.4.1 Overexpression of WT or A315T mutant TDP-43 is not overtly toxic to NSC-34 motor neuronal cells.

In an effort to clarify the discrepancy between TDP-43 loss-of-function or gain-of-function theories, we sought to delineate any possible gain of function toxic effects of TDP-43 overexpression. We transiently expressed GFP tagged-wild-type (WT) and A315T mutant TDP-43 in NSC-34 motor neuron/neuroblastoma hybrid cells. After 24 and 48 hours of expression, cell viability of GFP-expressing cells was established by assessing nuclear morphology for signs of cell death. As dividing cells display somewhat condensed nuclei, only those cells with clearly fragmented nuclei were counted as non-viable. There was no difference in toxicity observed in either A315T mutant or WT TDP-43 as compared to YFP-control transfected NSC-34 cells. GFP-tagged A315T and WT TDP-43 both had a principally nuclear localization and diffuse cytoplasmic staining, with somewhat punctate foci observed in both transfections (Fig 3.4.1A).

Although there was no overt morphologic toxicity observed due to overexpression of either form of TDP-43, it remained to be determined if overexpression rendered NSC-34 cells sensitive to cell stressors. Previous studies have established a correlation between proteasome inhibition and TDP-43 aggregation (van Eersel, et al., 2011). Furthermore, it has been found that conditional knock-out of proteasome subunit Rpt3 in mice resulted in ALS phenotype and ALS-like TDP-43 pathophysiology (Tashiro et al., 2012). As such, we sought to determine if overexpression of GFP-tagged WT or A315T

TDP-43 rendered NSC-34 cells more susceptible to proteasomal inhibition. After 48 hours of expression of GFP-control, WT or A315T TDP-43, NSC-34 cells were subsequently treated with 1 or 10 μ M of the proteasome inhibitor MG132 for 48 hours. Again, toxic effects were analyzed by assessing the nuclear morphology of transfected cells under the various treatment conditions. There was not a substantial increase in cell death observed with either GFP-tagged WT or A315T TDP-43 when treated with MG132 (as compared to GFP control vector), although a slight trend in increased cell death was observed at the higher MG132 concentration (Fig. 3.4.1 B).

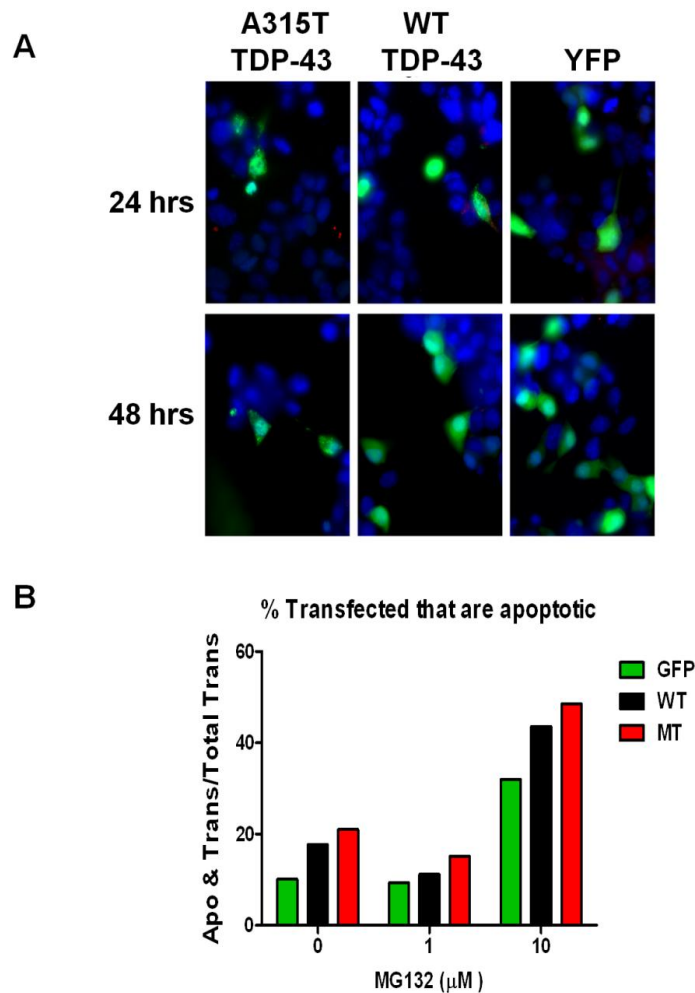


Fig. 3.4.1 - Overexpression of WT or A315T mutant TDP-43 is not overtly toxic to NSC-34 motor neuronal cells. (A) NSC-34 cells were transfected for 24 and 48 hours with GFP-tagged WT TDP 43, A315T TDP 43 and YFP control vectors. Nuclei are stained with DAPI. Exogenous vectors expressing GFP are shown to localize to the nucleus (blue) and cytoplasm. GFP-positive cells were evaluated for cell death by assessing nuclear morphology, as described in the results section. Values are given as a percent of total transfected (GFP-positive) cells that contain fragmented nuclei. There was no difference in toxicity observed in either A315T mutant or WT TDP-43 as compared to YFP-control vector. (B) NSC-34 cells were treated with 1 or 10 μ M of the proteasome inhibitor MG132 for 48 hours after 48 hours expression of GFP-control, WT or A315T TDP-43. Again, toxic effects were analyzed by assessing cell morphology of transfected cells under the various treatment conditions. There was not a substantial increase in cell death observed with either GFP-tagged WT or A315T TDP-43 when treated with MG132 (as compared to GFP-tagged control vector), although a slight trend in increased cell death was observed at the higher MG132 concentration (n=1).

3.4.2 Depletion of cellular TDP-43 is not toxic to NSC-43 cells

Based on literature describing that TDP-43 related neuronal cell death is a result of a loss-of-function of TDP-43, we sought to determine the effects of knock-down of TDP-43 in NSC-34 cells. Cells were transfected with either siRNA to TDP-43 or non-target siRNA for 72 hours. Efficacy of transfection and subsequent knockdown of TDP-43 was determined by western blotting, and revealed a marked 60% decrease in TDP-43 protein levels using TDP-43 targeted siRNA, as compared to non-target siRNA and mock-transfected controls (transfection protocol carried out without the use of an siRNA construct) (Fig 3.4.2 A). Importantly, noticeable knockdown of TDP-43 using the non-targeted siRNA was not apparent, as compared to mock-transfected controls, indicating that knockdown using the TDP-43 targeted siRNA was not due to other effects of transfection (3.4.2 A).

After establishing significant knockdown of TDP-43, an MTT cell viability assay was used to evaluate differences in cell survival between the different experimental conditions. There was no significant difference in cell survival when comparing the TDP-43 siRNA transfection versus the non-target transfection. Of note, a decrease in viability was apparent in both the TDP-43 siRNA and non-target siRNA, as compared to controls cells not exposed to any of the transfection conditions (non-transfected controls), indicating an inherent toxicity in the transfection procedure (3.4.2 B). This is consistent with what was observed microscopically, as both transfected conditions had a decrease in the number of cells that remained attached after the transfection period, while control cells remained confluent and appeared mitotically active (data not shown).

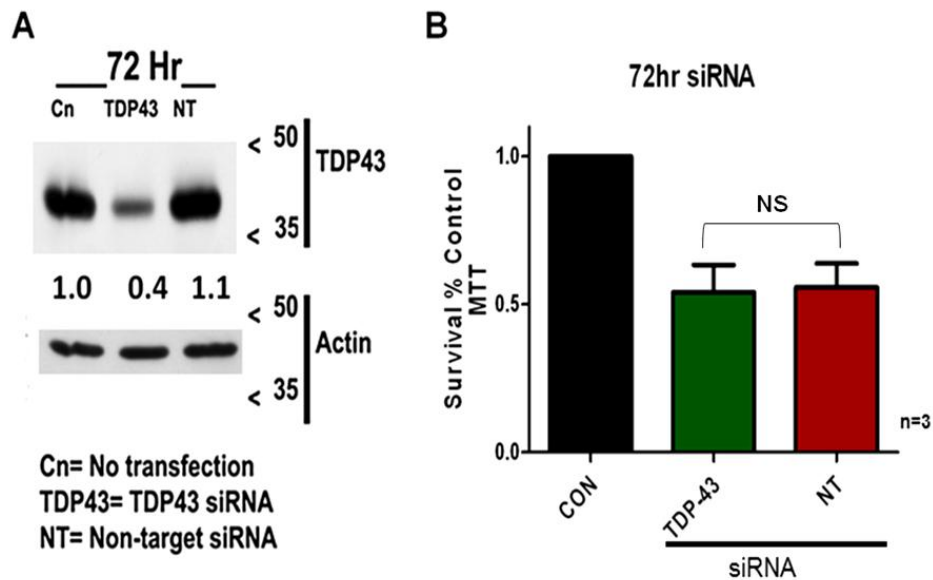


Fig. 3.4.2 - Depletion of cellular TDP-43 expression levels is not toxic to NSC-43 cells. NSC-34 cells were transfected with TDP-43 or non-target (NT) siRNA for 72 hours. (A) Efficacy of transfection was determined by western blotting. Relative TDP-43 protein levels are indicated relative to Actin control amounts. TDP-43 targeted siRNA caused a 60% decrease in TDP-43 protein levels (compared to non-target siRNA and mock-transfected controls). (B) Relative survival as determined by MTT cell viability assay. No significant difference in cell survival was observed when comparing the TDP-43 siRNA transfection versus the non-target transfection (n=3).

3.4.3 Endogenous and GFP-tagged TDP-43 partially localize to mitochondria

In order to better establish the effects of TDP-43 in NSC-34 cells, we transfected GFP-tagged WT and A315T TDP-43 and performed cell fractionation into mitochondrial and cytosolic fractions. Interestingly, immunoblotting revealed the presence of endogenous TDP-43 in all cell fractions examined, including the mitochondrial fraction. Moreover, transfected GFP-tagged WT and A315T TDP-43 also localized to the mitochondrial fractions (Fig. 3.4.3 A).

In order to establish that TDP-43 localization to mitochondria is not a phenomenon of the NSC-34 cell line, we performed cell fractionation on several other neuronal cell lines as well as primary neuronal and non-neuronal cultures. Interestingly, TDP-43 localization to the mitochondria occurred in all cell lines examined, specifically, HT22 hippocampal cell line as well as primary cerebellar granule neurons and primary astrocyte cultures (Fig. 3.4.3 B).

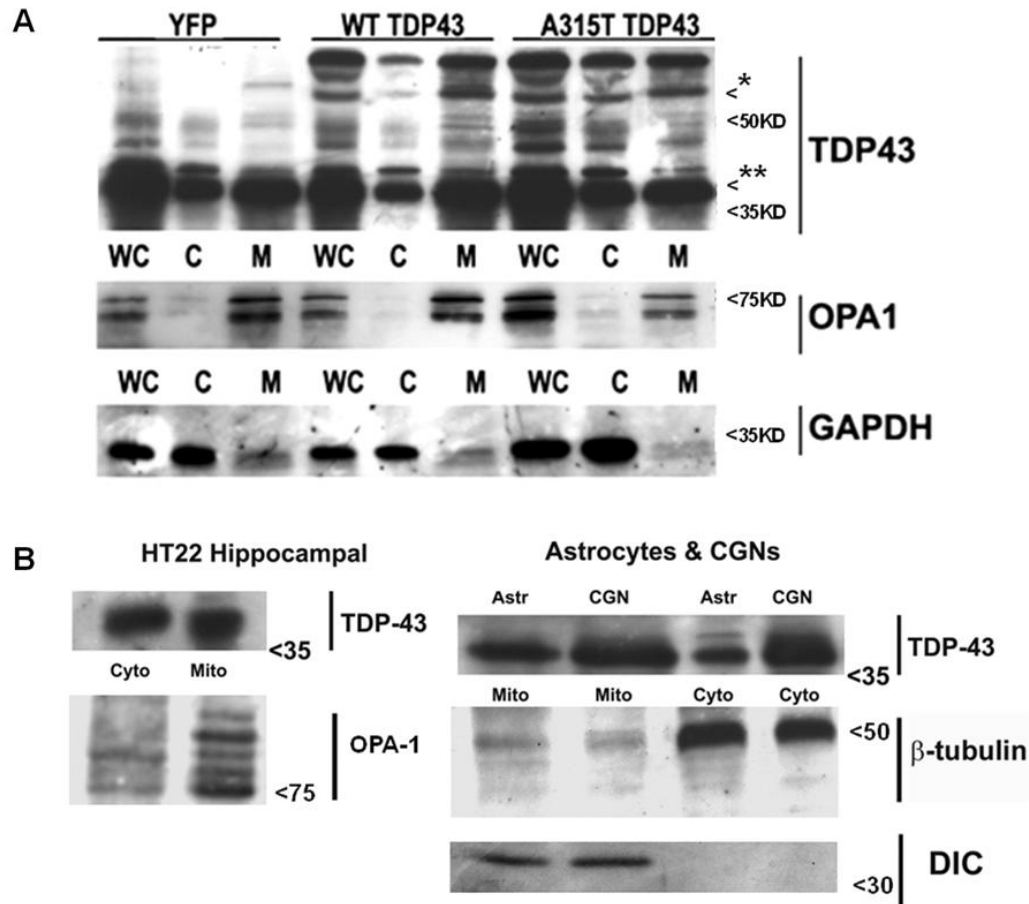


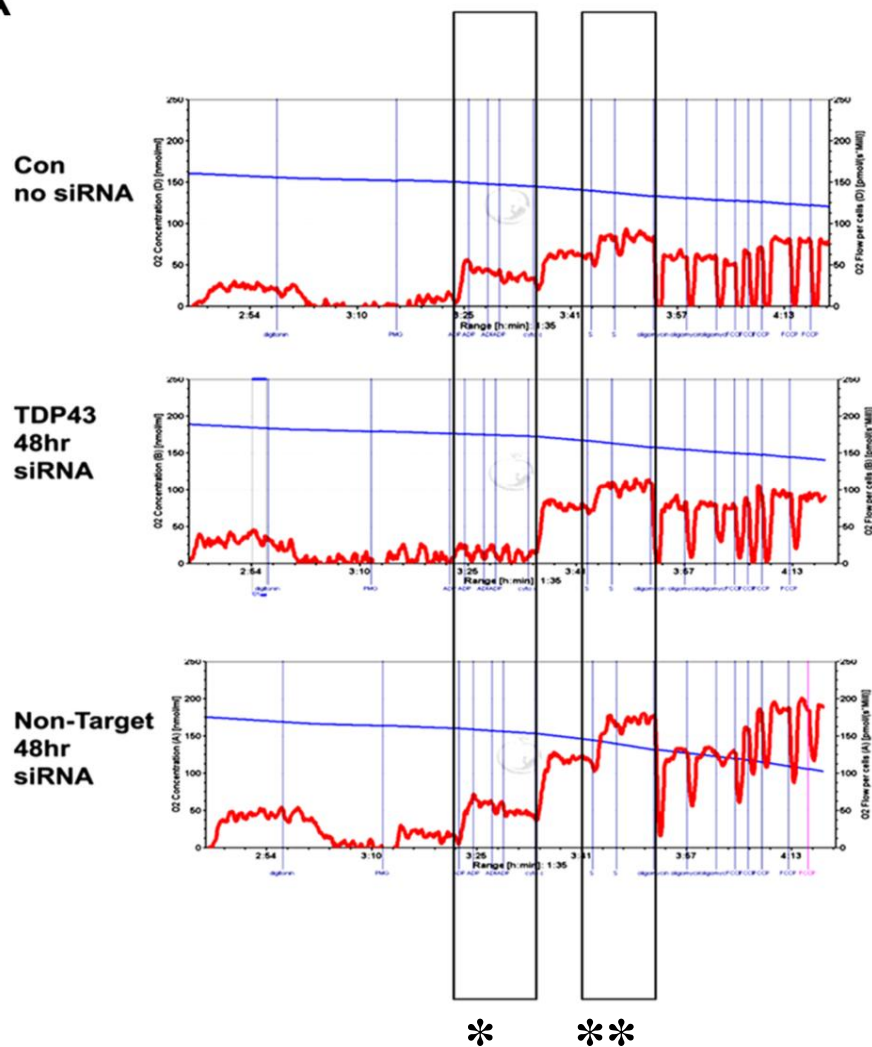
Fig. 3.4.3 - Endogenous and GFP-tagged TDP-43 partially localize to mitochondria. (A) NSC -34 cells were transfected with GFP-tagged WT and A315T TDP-43 and then subjected to cell fractionation. Immunoblotting revealed the presence of endogenous TDP-43 (**) in all cell fractions examined, including the mitochondrial fraction. GFP tagged WT and A315T TDP-43 (*) also localized to the mitochondrial fractions. Enrichment of mitochondrial and cytoplasmic fractions is confirmed by OPA1 and GAPDH. (WC=whole cell lysate; C=cytoplasmic fraction; M=mitochondrial fraction. (B) Cell fractionation and immunoblotting of non-transfected HT22 hippocampal cell line and astrocyte and CGN primary neuronal cultures also demonstrated endogenous TDP-43 localization to the mitochondria. Enrichment of mitochondrial fractions is confirmed by OPA1 in HT22 immunoblot and dicarboxylate carrier (DIC) in astrocyte/CGN immunoblot. β -tubulin indicates enrichment of cytoplasmic fraction in astrocyte/CGN immunoblot.

3.4.4 Knockdown of TDP-43 induces mitochondrial Complex I deficiency

In an effort to delineate the possible function of TDP-43 mitochondrial localization, we performed high-resolution respirometry using the *OROBOROS* Oxygraph-2k (O2k) system. NSC-34 cells were subjected to TDP-43 or non-target siRNA for 48 hours. After permeabilizing cells with digitonin, resting state (LEAK state) respiration was established in the presence of complex I substrates pyruvate, malate, and glutamate (PMG). Oxygen consumption under non-transfected and TDP-43 or non-target (NT) siRNA conditions remained equally low under these conditions. Upon initiation of oxidative phosphorylation (OXPHOS) through the addition of ADP, respiration was significantly less in TDP-43 siRNA treated cells as compared to non-transfected and NT siRNA treatments, indicating a defect in oxidative phosphorylation under these conditions. Of note, although NT treated cells had a higher respiration with the addition of ADP, it was visibly lower than what was observed in non-transfected controls. Again, this is likely due to the inherent toxicity from the transfection procedure. A surprising finding was that upon addition of the Complex II substrate, succinate, respiration in TDP-43 siRNA treated cells returned to what is seen in control/NT conditions. This is significant, as it effectively demonstrates that the observed OXPHOS deficiency can be overcome through bypassing Complex I. Respiration measurements with the addition of Complex V inhibitor, oligomycin, and the uncoupler FCCP remained similar under all experimental conditions (Figure 3.4.4 A). Statistical comparison of the respiration levels seen with addition of ADP, succinate and FCCP in all treatment conditions revealed a significant difference in Complex I function under TDP-43 siRNA conditions (Fig.3.4.4

B). Of note, whole cell lysates from each experimental condition were analyzed by immunoblotting to confirm that efficient TDP-43 knockdown (approximately 60%) was achieved (data not shown).

A



B

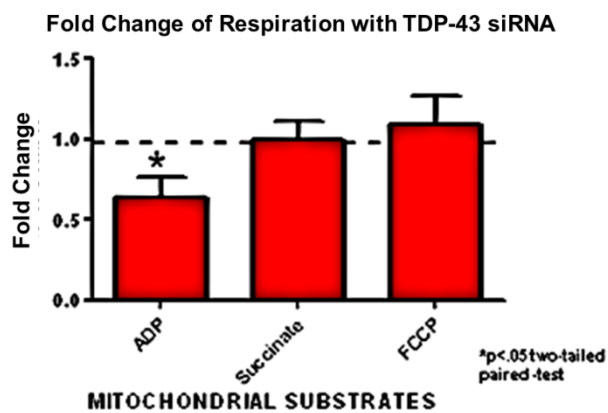


Fig. 3.4.4 - Knockdown of TDP-43 induces mitochondrial Complex I deficiency.

NSC3-34 cells were subjected to TDP-43 or non-target (NT) siRNA for 48 hours and analyzed for respiration efficiency using the *OROBOROS* Oxygraph-2k (O2k) high-resolution respirometer. Results were analyzed as compared to non-transfected control cells. (A) Resting state (LEAK state) respiration is comparable in all experimental conditions. Respiration is demonstrably less in TDP-43 siRNA treated cells as compared to non-transfected and NT siRNA treatments upon initiation of oxidative phosphorylation (OXPHOS) through the addition of ADP (*). Non-target siRNA treated cells display lower respiration than what is observed in non-transfected controls, indicating toxicity from the transfection procedure (*). Respiration in all three experimental conditions returns to comparable levels upon the addition of the Complex II substrate succinate (**). Respiration measurements are equivalent in all three experimental conditions during the addition of Complex V inhibitor, oligomycin, and the uncoupler FCCP. (B) Statistical comparison of respiration levels seen with addition of ADP, succinate, and FCCP in all treatment conditions reveals a significant difference in respiration levels during ADP addition to TDP-43 siRNA treated cells, (but not with addition of succinate or FCCP), as compared to non-transfected controls. * $p < 0.05$ paired Student's *t*-test, $n = 7$, error bar indicates SEM. Values shown are respiration levels of TDP-43 siRNA treated cells expressed as fold change of non-target siRNA treated cells.

3.5 Discussion

TDP-43 LOF and GOF models represent opposing ends on the spectrum of theories regarding the relationship between TDP-43 dysfunction and ALS/FTLD pathologies. Indeed, abundant evidence is all but conclusive that an underlying cause of these neurodegenerative diseases consists of atypical events involving this protein. However, the list of known TDP-43 functions is substantial and certainly not exhaustive, which has proven to be an added challenge in discerning putative gain-of-function or loss-of-function effects of TDP-43 dysfunction. Given these considerations, extrapolating the definitive mechanisms behind TDP-43 mediated neuronal cell death will undoubtedly be a complex process.

In the present study, we used *in vitro* overexpression and siRNA knockdown methods to approximate gain-of-function and loss-of-function effects, respectively. Each approach failed to elicit overt neuronal cell death or cause any morphological toxic effects. Overexpression data presented here were evaluated by assessing for apoptotic morphology in cells that were clearly expressing the GFP-tagged construct. Although this approach was seemingly prone to observer bias, other more objective methods (including flow cytometric analysis and cell viability assays) did not yield substantially different results. Furthermore, in order to account for transfection efficiency, visual assessment of each cell was necessary in order to consider only those cells that had been transfected. In contrast, siRNA studies utilized the MTT cell viability assay to more broadly assess for cell survival in TDP-43 siRNA treated cultures. Although we were able to substantially reduce TDP-43 expression levels (as evaluated by immunoblotting),

there was not considerable reduction in cell viability as a result of TDP-43 knockdown. Ultimately, despite multiple approaches and means of evaluating our data, we were not able to demonstrate TDP-43-mediated toxicity using these systems to model the effects of gain-of-function and loss-of-function.

The preceding findings, although seemingly inconclusive, are commensurate with the dichotomy in currently held theories surrounding the effects of TDP-43 loss-of-function or gain-of-function. Therefore, we considered this question in the broader context of mechanisms known to be involved in neurodegeneration and sought to evaluate if TDP-43 has any interaction with mitochondrial function. Surprisingly, it was noted that both endogenous and GFP-tagged TDP-43 (wild type or mutant) partially localized to mitochondrial fractions. This finding is significant because it established a previously unreported physical association between TDP-43 and the mitochondria, even under basal conditions.

To further characterize these findings, we examined mitochondrial function during conditions of decreased TDP-43 levels using high-resolution respirometry. Remarkably, TDP-43 siRNA treated cells demonstrated a deficit in mitochondrial oxidative phosphorylation. Further evaluation during these studies revealed that mitochondrial respiration returned to control levels when the system was able to bypass ETC Complex I by using by utilizing substrates (succinate) that are used by Complex II. Collectively, these findings demonstrate that significant mitochondrial complex I deficits occur upon depletion of cellular TDP-43 levels. These data are important in that they establish a relationship between TDP-43 and mitochondrial function. Until very recently,

this association had not been reported. Since we made this discovery, other reports have substantiated our findings. Transfected wild type, mutant, and TDP-43 C-terminal fragment localization to mitochondria has been validated through several studies utilizing cell fractionation, immunoblotting, and immunostaining (Hong et al., 2012; Wang et al., 2013). Importantly, Wang et al. (2013) was also able to establish that TDP-43 suppression in primary motor neurons had a deleterious effect on mitochondrial dynamics and function. Alternatively, overexpression studies have demonstrated deficits in mitochondrial morphology, membrane potential, and, activity of mitochondrial ETC Complex I (Hong et al., 2012; Lu et al., 2012; Wang et al., 2012). These overexpression studies are interesting when juxtaposed with knockdown studies, and indicate that mitochondrial health and function is susceptible to a fragile balance of TDP-43 levels.

TDP-43 functions are largely comprised of modulating gene expression through direct DNA and RNA binding. We propose that TDP-43 may be involved in regulation of mitochondrially encoded ETC components, and that disruptions in TDP-43 function result in a disturbance in the production of these essential proteins. Importantly, mitochondrial complex I proteins comprise the largest portion of protein coding genes within the mitochondrial genome. This is in accord with our discovery that TDP-43 deficiency results specifically in a deficit in the function of complex I.

We propose a model in which one of the endogenous functions of TDP-43 is to modulate expression of mitochondrially encoded ETC components. It is well established that ALS-associated cellular conditions, such as oxidative stress, can result in impaired mitochondrial membrane potential and disruption of mitochondrial respiration and

dynamics. We suggest that during these scenarios, nuclear export of TDP-43 is increased in order to facilitate increased regulation of mitochondrially encoded ETC components in an effort to offset potential damage incurred to mitochondrial components. In extreme circumstances, such as what is observed during neurodegeneration, nuclear export of TDP-43 is increased to levels that overwhelm cytoplasmic protein trafficking systems. Subsequently, TDP-43 aggregates form and become ubiquitinated by proteasome machinery in an effort to combat these effects. Future studies, including quantification of mitochondrially encoded Complex I mRNA levels, will be necessary to appropriately test our proposed model.

Chapter Four: TDP-43 nuclear export is partially mediated by mitochondrial glutathione levels and c-Jun N –Terminal Kinase

4.1 Abstract

TAR DNA-binding protein-43 (TDP-43) cytoplasmic inclusions are a consistent pathological finding in several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). Furthermore, mutations in TDP-43 are identified in both familial and sporadic forms of ALS, which underscores the significance of this protein in ALS etiology. Despite the preponderance of evidence linking TDP-43 dysfunction to neuronal cell death, little insight has been given into the regulation of TDP-43 nuclear export and cytoplasmic localization. In the present study, we report that TDP-43 nuclear export is enhanced by reductions in cellular glutathione (GSH) levels. This effect is inhibited by overexpression of the mitochondrial GSH transporter, the 2-oxoglutarate carrier (OGC), which specifically increases the mitochondrial GSH pool. In addition, inhibition of c-Jun N-terminal kinase (JNK) but not p38 MAP kinase effectively ameliorates EA-induced TDP-43 nuclear export. Our data offers insight into regulatory mechanism of TDP-43 cellular distribution and further emphasize a relationship between mitochondrial physiology, TDP-43 dysfunction, and neuronal cell death.

4.2 Introduction

TDP-43 dysfunction is a hallmark of several neurodegenerative diseases, such as, ALS and FTLTD (Neumann et al. 2006; Cairns et al., 2007). It is classified in the hnRNP family of proteins and is involved various aspects of gene expression through direct binding of RNA and DNA. Under normal conditions, TDP-43 is predominantly located in the nucleus, but is continuously imported and exported to perform various nuclear and cytoplasmic functions (Ayala et al., 2008; Winton et al., 2008). However, TDP-43 is shown to form cytoplasmic ubiquitin-positive inclusions in brain and spinal cord motor neurons of ALS patients (Neumann et al., 2006). Furthermore, it has been shown that these cytoplasmic aggregates contained hyperphosphorylated and cleaved TDP-43 fragments (Arai et al., 2006; Neumann et al., 2006 Cairns et al., 2007; Giordana et al., 2010). Importantly, increased cytoplasmic distribution of TDP-43 occurs in conjunction with decreased nuclear TDP-43 levels, which is indicative of TDP-43 loss of function in the nucleus. Despite the prevalence of these findings in disease processes, the mechanisms involved in TDP-43 cellular distribution are not well established.

Previously, we described that TDP-43 consistently localizes partially to mitochondria in diverse neuronal cell types, implying a potential link between TDP-43 proteinopathy and mitochondrial-dependent neuronal cell death. In addition, siRNA-mediated TDP-43 depletion was associated with a deficit in mitochondrial ETC complex I, establishing a correlation between TDP-43 function and mitochondrial physiology. In the present study, we report that TDP-43 nuclear export is enhanced by specific reductions in mitochondrial GSH levels in NSC-43 cells. This effect is inhibited by

stable overexpression of the mitochondrial GSH transporter, OGC, which specifically increases the mitochondrial GSH pool. Furthermore, inhibition of c-Jun N-terminal kinase (JNK) but not p38 MAP kinase effectively ameliorates EA- induced TDP-43 nuclear export. Our data suggest a novel method of regulating TDP-43 cellular distribution and further highlight a relationship between mitochondrial physiology, TDP-43 dysfunction, and neuronal cell death.

4.3 Materials and Methods

4.3.1 Materials

The monoclonal antibody to TDP-43 was obtained from ProteinTech (Chicago, IL, USA). The monoclonal antibody to OPA1 (residues 708–830) was obtained from BD Biosciences (San Diego, CA, USA). Mitochondrial/cytosolic fractionation kit was from Biovision (Mountain View, CA, USA), ethacrynic acid, β -tubulin, and GSH monoethylester were purchased from Sigma-Aldrich (St. Louis, MO, USA)., Geneticin (G418) was obtained from Invitrogen (Carlsbad, CA, USA). The GSH assay was purchased from Oxford Biomedical (Rochester Hills, MI, USA). The mitochondrial/cytosolic fractionation kit was from Biovision (Mountain View, CA, USA). Antibody against cytochrome *c* oxidase IV (Cox-IV) was purchased from Cell Signaling (Beverly, MA, USA). Anti-glyceraldehyde 3-phosphate dehydrogenase (Anti-GAPDH) was purchased from Abcam (Cambridge, MA, USA). Secondary antibodies for immunoblotting and reagents for enhanced chemiluminescence were obtained from GE Life Sciences (Piscataway, NJ, USA). DharmaFECT Transfection Kit, smart pool siRNA

against mouse TDP-43, and non-target siRNA were from ThermoScientific Pierce Biotechnology (Rockford, IL, USA). Optimem and Dulbecco's modified Eagle's medium were obtained from Gibco (Carlsbad, CA, USA). The V5-OGC plasmid used to create stably expressing NSC-34 cells was a generous gift from Dr. Larry Lash (Wayne State University). NSC34 cells were kindly provided by Dr. Neil Cashman (University of British Columbia).

4.3.2 Cell Culture

NSC-34 cells were plated at a concentration of 250,000 cells per well in a tissue culture treated 6-well culture vessel and maintained in high-glucose DMEM, 10% FBS, 1% pen-strep. OGC-overexpressing NSC-34 cells were established as described in Wilkins et al. (2014) and maintained through addition of 500 µg/mL of G418 in NSC-34 media.

4.3.4 Cell lysis and immunoblotting

After treatment as described in the Results section, cells were washed once in 1 mL ice-cold phosphate-buffered saline (PBS) (pH 7.4). Cells were incubated for 10 min on ice in lysis buffer (150–200 µl per 35-mm well) prepared as described previously (Loucks et al., 2006). The cells were then harvested by scraping and cell debris were removed by centrifugation at 6000 g for 2 min. Protein concentration was determined using BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) with final absorbance of samples measured by a microplate spectrophotometer (BioTek PowerWave XS2, Winooski, VT, USA). Whole cell lysates were subjected to SDS-PAGE through 7.5% polyacrylamide gels. Resolved proteins were transferred to PVDF

membranes and processed for immunoblot analysis as previously described (Loucks et al., 2006). In general, western blots shown are representative of a minimum of three independent experiments.

4.3.5 Immunofluorescence

After treatment as described in the Results section, cells were fixed for 1 h at room temperature in 4% paraformaldehyde followed by permeabilization and blocking in 0.2% Triton X-100 and 5% BSA in 1X PBS (pH 7.4) for one hour. Cells were incubated overnight in TDP-43 primary antibody and DAPI diluted in 2% BSA and 0.2% Triton X-100 in PBS. Cells were then washed five times in 1X PBS followed by a 1 hour incubation in FITC-conjugated secondary antibody diluted in 2% BSA and 0.2% Triton X 100 in PBS. Subsequent to washing five times in 1X PBS, cells were placed in anti-quench (5 mg/ml *p*-phenylenediamine in 1X PBS). Increased cytoplasmic TDP-43 levels were indicated by cells which maintained significant FITC TDP-43 fluorescence when control cytoplasmic FITC levels were decreased to undetectable amounts.

4.3.6 Cell Fractionation

Cells were treated as indicated in the results section. Media was aspirated and cells were washed once with 1X ice-cold phosphate-buffered saline (PBS), pH 7.4. One ml of cytosolic buffer provided in the kit was diluted 1 : 5 in ddH₂O, with added protease inhibitor cocktail and 1 mM dithiothreitol as per the manufacturer's recommendations. A 100-μL aliquot of the cytosolic buffer was added per well and incubated on ice for 20 min, at which point cells were harvested and homogenized with 60-80 passes of a Dounce pestle. Effective homogenization at this step was confirmed microscopically. A

whole cell lysate sample was collected at this point and added to wahl lysis buffer. Samples were then spun down at 720 g for 10 min at 4°C two times, with the supernatant removed and placed in a fresh tube each time. The supernatant was transferred to a fresh tube and spun tube down at 10 000 g for 30 min at 4°C to collect the mitochondrial pellet, which was resuspended in 100 µL of mitochondrial buffer (provided in the kit) with protease inhibitor cocktail and 1 mM dithiothreitol as per the manufacturer's recommendations. The remaining cytosolic supernatant was transferred to a fresh tube.. Samples were immediately used for further experiments or frozen down at -20 C.

4.3.7 Glutathione Assay

Glutathione levels [reduced GSH + oxidized glutathione (GSSG)] were measured using an assay kit (DTNB) from Oxford Biomedical, following the manufacturer's protocol. All glutathione measurements were normalized to protein concentration and performed on fresh lysates.

4.4 Results

4.4.1 Depletion of cellular GSH levels induces nuclear export of TDP-43

In an effort to establish possible triggers of TDP-43 nuclear export, we evaluated the effects of mitochondrial oxidative stress on the cellular distribution of TDP-43 in NSC-34 cells. Specifically, EA was used to deplete cellular GSH levels. Cells were exposed to 50 µM EA for 4 hours and subsequently stained for TDP-43. Microscopic imaging revealed that TDP-43 has a predominantly nuclear localization in control conditions. This is in accord with many studies that report TDP-43 exists primarily in the

nucleus. Upon EA treatment, TDP-43 begins to adopt a cytoplasmic localization along with a decrease in nuclear presence. To confirm that these observations are the result of EA effects on GSH levels, EA treatment was performed subsequent to 48 hour glutathione monoethylester (GSH-MEE) supplementation in order to restore cellular GSH levels. These studies reveal that EA-induced TDP-43 nuclear export is prevented upon restoration of GSH levels (Fig. 4.4.1 A). These findings are in accord with what has been recently reported by Iguchi et al. (2012).

We previously established that mitochondrial GSH levels are tightly coupled to oxidative stress induced neuronal cell death (Wilkins et al., 2013). In order to establish the relevance of that paradigm to our present findings, we measured GSH levels in cellular fractions of NSC-34 cells exposed to GSH-MEE. Interestingly, we reveal that treatment of NSC-34 cells with GSH-MEE selectively increases mitochondrial GSH levels to well over that of non-treated conditions (Fig. 4.4.1 B) (Wilkins et al., 2014). These data suggest that depletion of mitochondrial GSH levels specifically has an effect on TDP-43 cellular distribution.

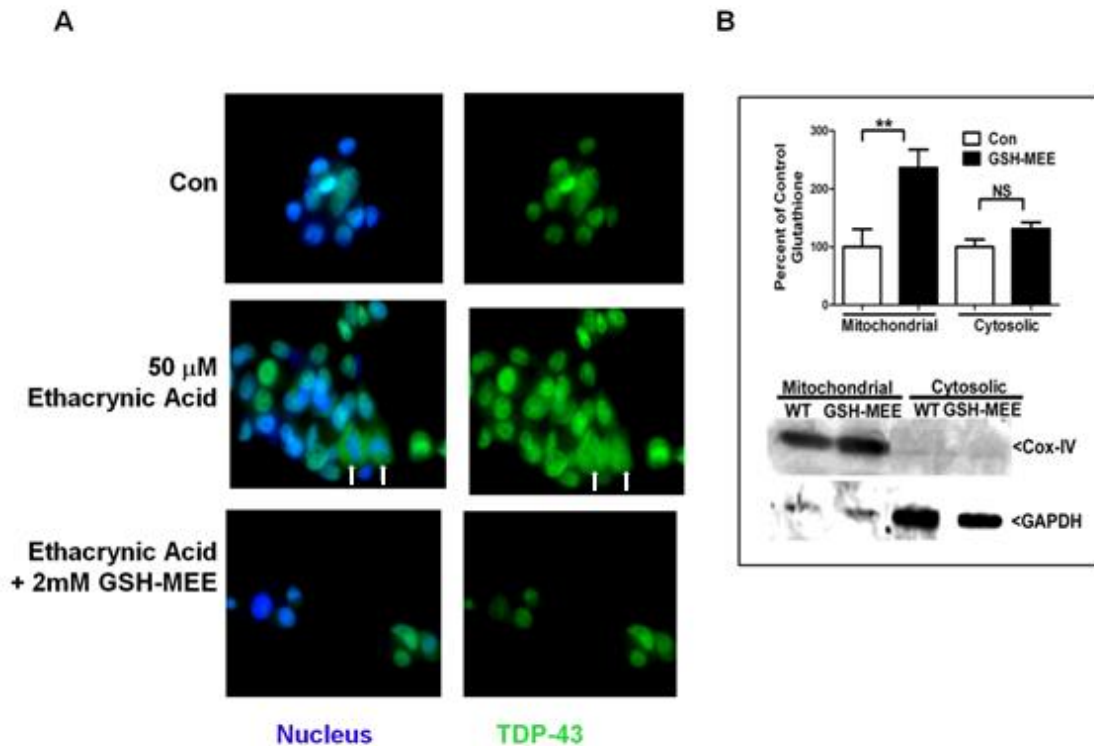


Fig. 4.4.1 - Depletion of cellular GSH levels induces nuclear export of TDP-43. (A) NSC34 cells were exposed to 50 μ M ethacrynic acid (EA) for 4 hours and subsequently stained for TDP-43 (green). Nuclei are stained with DAPI (blue) TDP-43 has a predominantly nuclear localization in control conditions. EA treatment induces nuclear export of TDP-43 along with an increase in cytoplasmic localization (indicated by arrows). Pre-treatment with GSH monoethylester (GSH-MEE) prevents redistribution of TDP-43 (n=3 independent experiments). (B) Quantification of GSH levels indicates that treatment of NSC-34 cells with GSH-MEE selectively increases mitochondrial GSH levels. Mitochondrial and cytosolic enrichment in cell fractions are indicated by COX-IV and GAPDH, respectively. **p < 0.01 versus control via an unpaired Student's *t*-test n = 7, error bar indicates SEM (as published in Wilkins et al., 2014). Acknowledgments are given to Heather M. Wilkins, PhD and Samantha Brock for their contribution to these experiments.

4.4.2 Stable expression of the mitochondrial OGC carrier specifically increases mitochondrial GSH levels.

To better characterize the role of mitochondrial GSH in nuclear export of TDP-43, we utilized an NSC-34 cell line established in our lab that stably expresses the mitochondrial inner membrane transporter OGC. We recently reported that OGC is responsible for mitochondrial uptake of GSH via direct interactions with BCL2 in neurons (Wilkins et al., 2014). These studies reveal that NSC-34 cells that stably overexpress this carrier have a marked and specific increase in mitochondrial GSH levels over that of control NSC-34 cells (Fig. 4.4.2 A). In the context of EA depletion, it was also determined that these cells maintain mitochondrial GSH levels well above that of control cells during EA treatment, while cytosolic GSH levels remained similarly reduced between both cell lines (Fig. 4.4.2 B) (Wilkins et al., 2014).

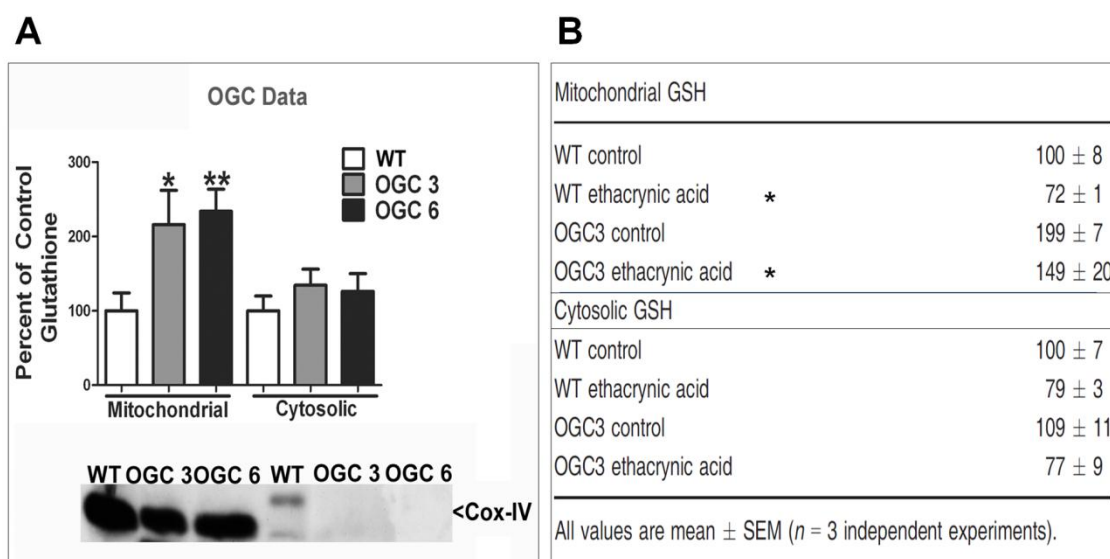


Fig 4.4.2 - Stable expression of the mitochondrial 2-oxoglutarate effectively increases mitochondrial GSH levels. (A) Cell fractionation and subsequent measurement of GSH levels in two different NSC-34 cell lines stably overexpressing the mitochondrial 2-oxoglutarate carrier (OGC3, OGC6) demonstrate a significant increase in mitochondrial GSH levels over that of WT NSC-34 cells. Enrichment of mitochondrial fraction is indicated by COX IV immunoblot. Mitochondrial data are represented as a percentage of WT mitochondrial GSH measurements. * $p < 0.05$, ** $p < 0.01$, unpaired Student's t -test, $n=7$ independent experiments, error bars indicate SEM. Cytosolic data are represented as a percentage of WT cytosolic GSH measurements. $n=7$ independent experiments, error bars indicate SEM. (B) OGC3 cell line maintains mitochondrial GSH levels above that of control cells. Data are represented as a percentage of WT control mitochondrial GSH measurements. Cytosolic GSH levels remain similar between all cell types. Data are represented as a percentage of WT control cytosolic GSH levels. * $p < 0.05$, $n=3$ independent experiments, error bars indicate SEM. (As published by Wilkins et al., 2014).

Acknowledgments are given to Heather M. Wilkins, PhD and Samantha Brock for their contribution to these experiments.

4.4.3 Stable overexpression of the mitochondrial OGC carrier prevents nuclear export of TDP-43.

Mitochondrial GSH levels were effectively sustained during EA treatment in OGC overexpressing NSC-34 cells. In contrast, cytosolic GSH levels decreased to that of WT cells. Thus, it can be said that the OGC cell line, when challenged with EA, experiences a decrease in cellular GSH levels while maintaining mitochondrial GSH levels. In consideration of this, we further investigated TDP-43 cellular distribution under this experimental paradigm. Under control conditions, WT and OGC overexpressing cells both display TDP-43 localization to the nucleus, with a slight cytoplasmic presence. However, upon treatment with EA, WT cells develop an increase in TDP-43 cytoplasmic distribution. This is in stark contrast to the OGC cells, which maintain a nuclear TDP-43 distribution (Fig. 4.4.3 A). Quantification of the number of cells displaying a marked cytoplasmic presence of TDP-43 reveals that OGC overexpression essentially prevents the redistribution of TDP-43 induced by EA in WT cells (Fig. 4.4.3 B). These data further indicate that specific decreases in mitochondrial GSH levels trigger nuclear export of TDP-43.

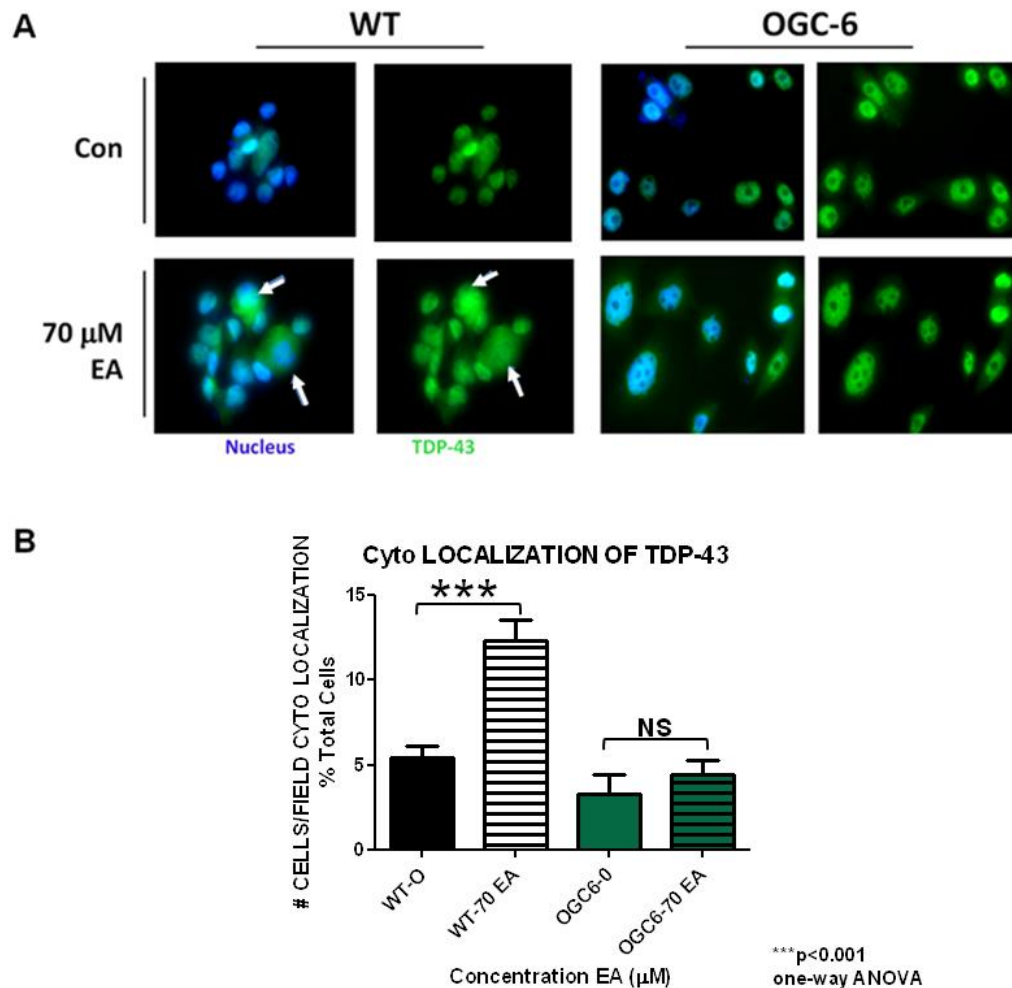


Fig. 4.4.3 - Stable overexpression of the mitochondrial 2-oxoglutarate carrier prevents nuclear export of TDP-43. (A) WT and OGC overexpressing cells were treated with 70 μ M EA for 4 hours followed by staining and imaging. TDP-43 is indicated in green and nuclei are indicated in blue (DAPI). Both cell lines display TDP-43 localization to the nucleus and a slight cytoplasmic presence. Upon treatment with EA the WT cell line develops an increase in TDP-43 cytoplasmic distribution, as indicated by arrows, while the OGC cell line maintains nuclear TDP-43 levels. (B) Quantification of the number of cells displaying an increase in cytoplasmic TDP-43 levels. EA acid treatment causes a significant increase in cytoplasmic levels of TDP-43 in WT cells, as compared to non-treated WT cells. OGC6 cytoplasmic levels during EA treatment are not significantly different than control non-treated OGC6 cells. ***p<0.001, NS = not significant via a one-way ANOVA with *post hoc* Tukey's test, n=5, error bars indicate SEM.

4.4.4 Nuclear export of TDP-43 is ameliorated by inhibition of JNK but not p38 MAP kinase.

It is accepted that JNK and p38 MAP kinase pathways are major contributors to cell stress responses during oxidative stress-induced apoptosis (Davis, 2000). As such, we examined the effects of inhibiting these pathways during EA induced mitochondrial oxidative stress conditions. WT NSC-34 cells were exposed to a 1 hour pre-treatment with either a JNK inhibitor (SP600125) or a p38 MAP kinase inhibitor (SB203580), followed by a 4 hour EA treatment and analyzed for TDP-43 cytoplasmic localization. Interestingly, pretreatment with the JNK inhibitor significantly attenuates EA induced TDP-43 nuclear export. In contrast, inhibition of p38 MAP kinase does not seem to have any effect on TDP-43 nuclear export (Fig.4.4.4 A-B). These data indicate that oxidative stress-induced cell signaling via JNK is involved in TDP-43 cellular distribution induced by specific depletion of mitochondrial GSH.

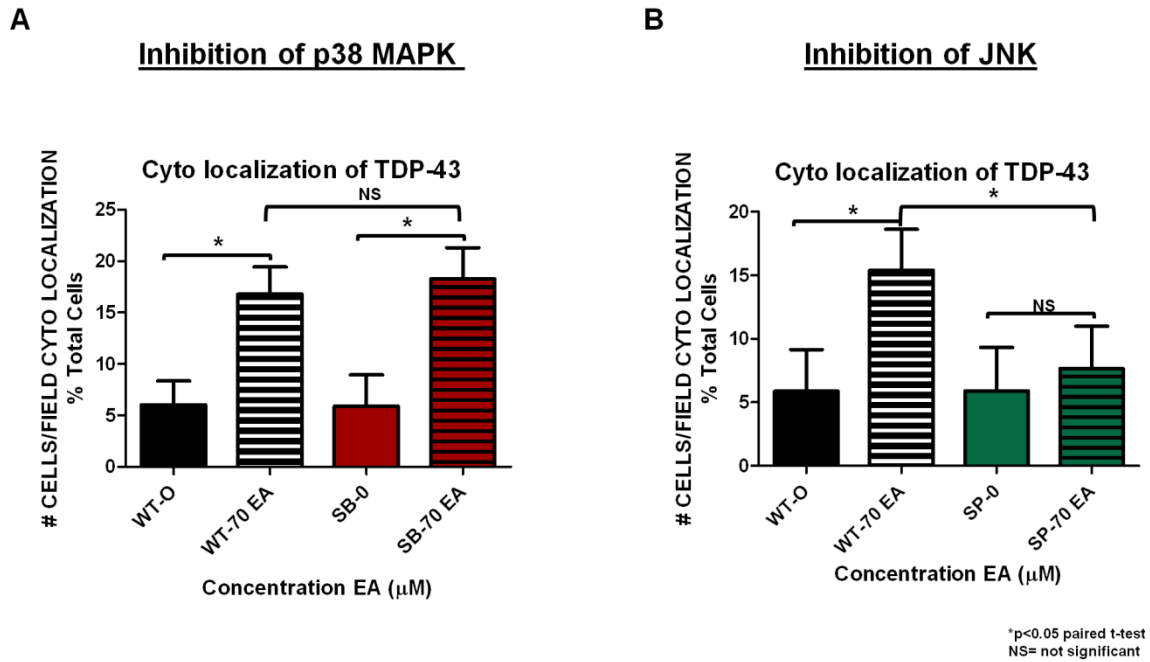


Fig 4.4.4 - Nuclear export of TDP-43 is ameliorated by inhibition of JNK but not p38 MAP kinase. Prior to EA treatment, WT NSC-34 cells were pre-treated for 1 hour with JNK inhibitor (SP600125) or p38 MAP kinase inhibitor (SB203580) and analyzed for TDP-43 cytoplasmic localization. (A) p38 MAP kinase (SB203580) does not have a significant effect on TDP-43 nuclear export. (B) JNK inhibitor (SP600125) significantly attenuates EA induced TDP-43 nuclear export. * $p < 0.05$, paired Student's *t*-test, $n=3$ independent experiments, error bars indicate SEM.

4.5 Discussion

Protein aggregation is a common pathogenic mechanism in diverse neurodegenerative disorders. TDP-43 cytoplasmic inclusions are pathological hallmarks of several neurodegenerative diseases, including ALS and FTLT. Furthermore, TDP-43 mutations have been identified in both familial and sporadic forms of ALS. Under typical conditions, TDP-43 exists primarily in the nucleus, but is also continuously translocated between the nucleus and cytoplasm (Ayala et al., 2008; Winton et al., 2008). In our previous studies, we corroborated recent findings that TDP-43 partially localizes to the mitochondria in diverse neuronal cell types. Despite these findings being established by other labs, there is still very little knowledge about the cell signaling that controls TDP-43 cellular distribution.

Iguchi et al. recently reported that depletion of cellular GSH levels induces TDP-43 cytoplasmic distribution (Iguchi et al., 2012). These findings are some of the first to establish a possible mechanism that induces TDP-43 nuclear export. Moreover, unpublished data from our lab demonstrate that mitochondrial GSH levels are reduced in an ALS mouse model spinal cord tissue of transgenic ALS mice, which further substantiates the correlation between mitochondrial GSH levels and mitochondrial mediated neuronal cell death. As such, we sought to delineate the role of mitochondrial GSH levels in TDP-43 nuclear export. We report that TDP-43 nuclear export is enhanced by specific reductions in mitochondrial GSH levels in NSC-34 cells. Similarly, redistribution of TDP-43 from the nucleus to the cytoplasm is induced by EA depletion of cellular GSH levels. This effect is inhibited by stable overexpression of the

mitochondrial GSH transporter, OGC, which specifically increases the mitochondrial GSH pool. Furthermore, inhibition of c-Jun N-terminal kinase (JNK) but not p38 MAP kinase effectively ameliorates EA induced TDP-43 nuclear export. Our data suggest a novel mechanism of regulating TDP-43 cellular distribution and further highlights a relationship between mitochondrial physiology, TDP-43 dysfunction, and neuronal cell death. These findings provide insight into possible triggers of TDP-43 nuclear export, and couples very specific redox events to the almost universal finding of increased TDP-43 export to the cytoplasm in neurodegenerative diseases. Further studies are needed to definitively establish a direct correlation between JNK signaling and TDP-43 cellular localization. As such, we propose to ascertain the effects of overexpressing constitutively active JNK in these experimental paradigms, and hypothesize that this will induce nuclear export of TDP-43.

-

Chapter Five: Conclusion

Mitochondrial dysfunction is considered an integral component of cell death pathways involved in neurodegeneration. As such, investigations into the origins and effects of these processes remain a fundamental component in determining possible therapeutic interventions. Mitochondria can elicit cell death through release of cytochrome *c* and subsequent initiation of the apoptotic cascade, which is tightly coupled to disruptions in mitochondrial function as well as dysfunctions in the mitochondrial fission-fusion equilibrium. Both conditions consistently occur in various neurodegenerative diseases; however, the cause and effect relationships remain controversial. Thus, the purpose of the investigations presented here are to provide further insight into novel sources of disturbances in mitochondrial form and function, as well as correlate these events to other mechanisms that are coupled to the etiology of neurodegeneration.

5.1 Summary of major findings

The major goal of chapter two was to further characterize an N-terminal cleavage of the mitochondrial fusion protein, OPA1. Initial studies in primary neuronal cultures described this processing as being mediated *indirectly* by caspases under classical apoptotic conditions, resulting in a previously unreported truncation product. Moreover, N-terminal cleavage of OPA1 occurs with concomitant fragmentation of the mitochondrial network and release of cytochrome *c* (Loucks et al., 2009). As presented

in this thesis, it was subsequently discovered that an identical N-terminal OPA1 cleavage product occurred in neurons as a result of oxidative and nitrosative stress. However, OPA1 processing under these conditions occurred in a caspase-*independent* manner. Of note, an identical N-terminal cleavage product was also demonstrated to occur spontaneously in aged neural tissue (Gray et al., 2013). Collectively, these findings establish that this N-terminal cleavage of OPA1 is coupled to mitochondrial dysfunction, and occurs in oxidative and nitrosative stress environments that are consistently linked to both aging and the pathogenesis of neurodegenerative diseases.

Studies performed in chapters three and four sought to elucidate the role of TDP-43 in mitochondrial dysfunction and neuronal cell death. TDP-43, an hnRNP, is found in ubiquitin-positive cytoplasmic inclusions in several neurodegenerative diseases, including ALS. However, little insight has been obtained into how dysregulation of this protein is involved in disease processes, and until very recently, TDP-43 involvement with mitochondrial function had not been identified. Chapter three demonstrated that neither overexpression of WT or A315T mutant TDP-43 had discernible toxic effects on NSC-34 cells. In addition, effective siRNA knockdown of TDP-43 also failed to induce cell death. It was noted that TDP-43 localized to mitochondrial fractions in NSC-34 and HT22 hippocampal cell lines, as well as primary cultures of astrocytes and cerebellar granule neurons, suggestive of a novel function for TDP-43 at the mitochondria. Interestingly, a very significant discovery was that siRNA depletion of TDP-43 resulted in a notable deficiency in mitochondrial ETC Complex I. These findings provide an

important mechanistic connection between TDP-43 dysfunction and mitochondrial impairment.

In an effort to further delineate these findings, the studies in chapter four sought to examine possible mechanisms involved in TDP-43 nuclear export. We were able to demonstrate that TDP-43 nuclear export is enhanced by reductions in mitochondrial GSH levels in NSC-34 cells. This effect is inhibited by stable overexpression of the mitochondrial GSH transporter, the 2-oxoglutarate carrier (OGC), which specifically increases the mitochondrial GSH pool. Furthermore, inhibition of c-Jun N-terminal kinase (JNK) also inhibited ethacrynic acid (EA)-induced TDP-43 nuclear export. These data indicate regulatory mechanisms of TDP-43 cellular distribution and further highlight a relationship between mitochondrial physiology, TDP-43 dysfunction, and neuronal cell death.

5.2 Discussion

Neuronal cell death underlies several devastating neurodegenerative diseases. It is consistently substantiated in literature that mitochondrial dysfunction resulting from oxidative or nitrosative stress can initiate neuronal cell death via apoptosis or necrosis. Accordingly, apoptotic cell death pathways are mechanistically linked to disruptions in the mitochondrial fission and fusion equilibrium, which is maintained through an intricate balance in expression levels and protein processing of the mitochondrial dynamics machinery. As such, in order to completely understand the role of mitochondrial dysfunction in the process of neuronal cell death, it is crucial to evaluate how this delicate fission and fusion equilibrium is maintained and disturbed.

OPA1 is a dynamin GTPase whose main function is to facilitate fusion of the mitochondrial innermembrane. In addition, OPA1 performs the fundamental tasks of maintaining mitochondrial cristae architecture and sequestering cytochrome *c* within the mitochondrial intermembrane space through homo-oligomerization (Cipolat et al., 2004; Frezza et al., 2006). Further understanding of OPA1 regulation is essential to clarifying the link between disruption of mitochondrial fission/fusion and induction of apoptosis.

OPA1 function is largely regulated through post-translational proteolytic processing to create long and short isoforms. These isoforms interact to form functional OPA1 oligomers that promote mitochondrial fusion and cristae architectural integrity (Landes et al., 2010a; Landes et al., 2010b). We have previously revealed a caspase-mediated OPA1 cleavage event that results in the formation of an N-terminal truncated “short” form of OPA1. Importantly, this isoform lacks a critical residue, K301, within the GTPase domain, rendering it unable to effectively perform its necessary functions. Moreover, it represents a shift in the mitochondrial fission/fusion equilibrium. In the studies presented here, we reveal an identical OPA1 cleavage event that occurs *independent* of caspases during oxidative and nitrosative stress-induced cell death in CGNs. Notably, we also demonstrate that this OPA1 cleavage product occurs spontaneously in midbrain and hippocampal tissues obtained from aged mice and rats, suggesting that OPA1 cleavage may provide a point of convergence between oxidative stress, mitochondrial dysfunction, and aging; a mechanistic link that may be particularly relevant to the enhanced risk of neurodegeneration associated with advancing age.

We propose that two distinct mechanisms converge to activate the mitochondrial protease responsible for the N-terminal cleavage of OPA1. The first mechanism involves activation of a yet unknown OPA1 protease by a caspase during classical apoptotic conditions. This could occur by caspase-mediated cleavage of an inhibitory regulatory domain, for example. Subsequently, this protease then cleaves OPA1 at a novel N-terminal cleavage site. The second mechanism, observed under conditions of oxidative or nitrosative stress, involves activation of the unidentified OPA1 protease through a caspase-independent mechanism. This may occur via direct oxidative or nitrosative modification of the protease, for example, via S-nitrosylation. Further studies are necessary in order to identify the mitochondrial protease involved in producing this novel N-terminal cleavage product.

These findings are particularly relevant to the study of neurodegenerative disorders. Many studies substantiate the impact of pathogenic proteins on the mitochondrial fission and fusion machinery in various disease states. For instance disruptions in the mitochondrial fission and fusion equilibrium have been reported to occur in conjunction with proteinopathies that are found in AD, Huntington's disease, and PD. Collectively, these studies indicate that aberrant activation or suppression of the mitochondrial fission or fusion machinery plays a significant pathogenic role in a number of neurodegenerative disorders. In this regard, the OPA1 cleavage event described in this thesis has recently been reported to occur in a manganese (Mn)-induced Parkinsonism model (Alaimo et al., 2014). Interestingly, this event was commensurate with increased DRP1 localization to the mitochondria, further highlighting a potential link between

aberrant OPA1 processing and a shift in the fission-fusion equilibrium. Given these findings, it will be interesting to determine if aberrant processing of OPA1, particularly the generation of the N-terminal truncated cleavage product described here, is observed in disorders such as AD, PD, and Huntington's disease.

In addition to mitochondrial dysfunction being a consistent finding in neurodegeneration, protein aggregation is also a common pathogenic mechanism in many neurodegenerative disorders. Specifically, TDP-43 cytoplasmic inclusions are pathological hallmarks of several neurodegenerative diseases, including ALS and FTL. Moreover, implications of this protein's involvement in the etiology of neurodegenerative diseases are widespread, as indicated by identification of TDP-43 mutations in both familial and sporadic forms of ALS. TDP-43, an hnRNP, has been consistently demonstrated to be involved at various levels of gene expression, although many functions of TDP-43 remain to be determined. Under typical conditions, TDP-43 exists primarily in the nucleus, but is also continuously translocated between the nucleus and cytoplasm (Ayala et al., 2008; Winton et al., 2008). However, very little is known about the cell signaling that controls TDP-43 cellular distribution. Furthermore, although there are inherent implications that dysfunction of this protein has a causal role in neuronal cell death, the mechanism of toxicity remains to be determined.

LOF and GOF theories are both proposed models for TDP-43 mediated cell death in ALS/FTLD. Indeed, substantial evidence exists supporting that atypical events surrounding this protein are an underlying cause of these neurodegenerative diseases. However, the list of known TDP-43 functions is substantial and certainly not exhaustive,

which proves to be an added challenge in discerning putative GOF or LOF effects of TDP-43 dysfunction. Given these considerations, understanding the definitive mechanisms behind TDP-43 mediated neuronal cell death will undoubtedly be a complex process.

In the studies presented in this thesis, we used overexpression and siRNA knockdown methods to approximate GOF and LOF effects *in vitro*. Each approach failed to elicit overt neuronal cell death or cause any morphological toxic effects. However, these findings are not surprising, given the conflicting reports on this topic, and perhaps further substantiate the multi-faceted nature of TDP-43 mediated toxicity. We were able to demonstrate that WT and mutant TDP-43 partially localize to the mitochondria, which became substantiated in several other studies (Hong et al., 2012; Wang et al., 2013). It has also been reported that impairments in mitochondrial dynamics caused by overexpression of TDP-43 (WT or mutant), can be rescued through overexpression of the mitochondrial fusion protein MFN2, which establishes a novel link between TDP-43 function and mitochondrial dynamics (Wang et al., 2013). Additionally, we also demonstrated significant deficits in mitochondrial Complex I upon depletion of cellular TDP-43 levels. Interestingly, other studies demonstrate that overexpression of WT or mutant TDP-43 cause a defect in mitochondrial Complex I activity (Hong et al., 2012; Lu et al., 2012). Furthermore, dysfunctions in mitochondrial dynamics, membrane potential and cristae morphology have been observed upon depletion of TDP-43 (Wang et al. 2013). In lieu of similar phenotypes resulting from both overexpression and depletion of TDP-43, it is an interesting concept that perhaps mitochondrial health and function is

susceptible to a fragile balance of TDP-43 levels and function. Overall, these recent findings are noteworthy in that they establish a relationship between TDP-43 and mitochondrial function.

TDP-43 functions are largely comprised of modulating gene expression through direct DNA and RNA binding. We propose that TDP-43 may be involved in regulation of mitochondrially encoded ETC components, and that disruption in TDP-43 function results in a disturbance in the production of these essential proteins. We suggest that nuclear export of TDP-43 is triggered as a means to regulate transcription of damaged mitochondrial components, and that nuclear export of TDP-43 during mitochondrial catastrophe (as is observed during neurodegeneration) is increased to levels that overwhelm cytoplasmic protein trafficking systems. Subsequently, TDP-43 aggregates form and become ubiquitinated by proteasome machinery in an effort to combat these inclusions. In the context of mutant TDP-43 effects, we surmise that dysfunctional forms of TDP-43 are not able to properly participate in repair of damaged ETC components. This deficiency would likely cause subsequent increases in ROS production, causing further damage to the mitochondria. Interestingly, a recent study supports this theory, in which mice containing a heterozygous mutant TDP-43 knock-in (A315TKi) developed insoluble TDP-43 protein aggregates and disruptions in mitochondrial cristae architecture (Stribl et al., 2014), suggesting that expression of mutant TDP-43 at physiological levels results in TDP-43 histopathology and impairment in mitochondrial structure. Heterozygous TDP-43(A315TKi) mutants lost 10% of their body weight and developed

insoluble TDP-43 protein starting as early as 3 months after birth, a pathology that was exacerbated with age.

Finally, we report that TDP-43 nuclear export is enhanced by reductions in mitochondrial glutathione (GSH) levels in NSC-34 cells. This event is largely prevented by specifically increasing the mitochondrial GSH pool through stable overexpression of the mitochondrial GSH transporter OGC. In addition, we demonstrate that, inhibition of c-Jun N-terminal kinase (JNK), but not p38 MAP kinase, effectively ameliorates EA-induced TDP-43 nuclear export, thus implicating specific cell signaling factors that are involved in GSH depletion-mediated TDP-43 nuclear export. Moreover, other studies have demonstrated that oxidative stress-induced TDP-43 localization to cytoplasmic stress granules (SGs) preceded ubiquitin-positive TDP-43 aggregations, and that SG formation, but not TDP-43 cytoplasmic localization, was inhibited by incubation with a JNK inhibitor (Meyerowitz,et al., 2011; Parker et al., 2012). In contrast to our reports, TDP-43 nuclear export was not affected by JNK, yet aggregate formation, once in the cytoplasm, was affected by JNK signaling. These differences in the regulation of nuclear export by JNK may relate to the different insults used to induce oxidative stress; we used EA-induced GSH depletion whereas other groups used paraquat, which has a much more complex mode of inducing oxidative damage. Collectively, these data indicate a complex relationship between TDP-43 cellular localization and JNK signaling. Furthermore, our findings suggest a novel method of regulating TDP-43 cellular distribution and further highlight a relationship between mitochondrial physiology, TDP-43 dysfunction, and neuronal cell death.

References

- Abramov, A.Y., Canevari, L., Duchen, M.R., 2003. Changes in intracellular calcium and glutathione in astrocytes as the primary mechanism of amyloid neurotoxicity. *J Neurosci.* 23, 5088-95.
- Acharya, K.K., Govind, C.K., Shore, A.N., Stoler, M.H., Reddi, P.P., 2006. cis-requirement for the maintenance of round spermatid-specific transcription. *Dev Biol.* 295, 781-90.
- Aijaz, S., Erskine, L., Jeffery, G., Bhattacharya, S.S., Votruba, M., 2004. Developmental expression profile of the optic atrophy gene product: OPA1 is not localized exclusively in the mammalian retinal ganglion cell layer. *Invest Ophthalmol Vis Sci.* 45, 1667-73.
- Akepati, V.R., Muller, E.C., Otto, A., Strauss, H.M., Portwich, M., Alexander, C., 2008. Characterization of OPA1 isoforms isolated from mouse tissues. *J Neurochem.* 106, 372-83.
- Aksenov, M.Y., Tucker, H.M., Nair, P., Aksenova, M.V., Butterfield, D.A., Estus, S., Markesbery, W.R., 1998. The expression of key oxidative stress-handling genes in different brain regions in Alzheimer's disease. *J Mol Neurosci.* 11, 151-64.
- Alaimo, A., Gorjod, R.M., Beauquis, J., Munoz, M.J., Saravia, F., Kotler, M.L., 2014. Deregulation of mitochondria-shaping proteins Opa-1 and Drp-1 in manganese-induced apoptosis. *PLoS One.* 9, e91848.
- Alavi, M.V., Bette, S., Schimpf, S., Schuettauf, F., Schraermeyer, U., Wehrl, H.F., Ruttiger, L., Beck, S.C., Tonagel, F., Pichler, B.J., Knipper, M., Peters, T., Laufs, J., Wissinger, B., 2007. A splice site mutation in the murine Opa1 gene features pathology of autosomal dominant optic atrophy. *Brain.* 130, 1029-42.
- Alavi, M.V., Fuhrmann, N., Nguyen, H.P., Yu-Wai-Man, P., Heiduschka, P., Chinnery, P.F., Wissinger, B., 2009. Subtle neurological and metabolic abnormalities in an Opa1 mouse model of autosomal dominant optic atrophy. *Exp Neurol.* 220, 404-9.
- Alexander, C., Votruba, M., Pesch, U.E., Thiselton, D.L., Mayer, S., Moore, A., Rodriguez, M., Kellner, U., Leo-Kottler, B., Auburger, G., Bhattacharya, S.S., Wissinger, B., 2000. OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28. *Nat Genet.* 26, 211-5.
- Amador-Ortiz, C., Lin, W.L., Ahmed, Z., Personett, D., Davies, P., Duara, R., Graff-Radford, N.R., Hutton, M.L., Dickson, D.W., 2007. TDP-43 immunoreactivity in hippocampal sclerosis and Alzheimer's disease. *Ann Neurol.* 61, 435-45.
- Amati-Bonneau, P., Valentino, M.L., Reynier, P., Gallardo, M.E., Bornstein, B., Boissiere, A., Campos, Y., Rivera, H., de la Aleja, J.G., Carroccia, R., Iommarini, L., Labauge, P., Figarella-Branger, D., Marcorelles, P., Furby, A., Beauvais, K., Letournel, F., Liguori, R., La Morgia, C., Montagna, P.,

- Liguori, M., Zanna, C., Rugolo, M., Cossarizza, A., Wissinger, B., Verny, C., Schwarzenbacher, R., Martin, M.A., Arenas, J., Ayuso, C., Garesse, R., Lenaers, G., Bonneau, D., Carelli, V., 2008. OPA1 mutations induce mitochondrial DNA instability and optic atrophy 'plus' phenotypes. *Brain*. 131, 338-51.
- Amati-Bonneau, P., Milea, D., Bonneau, D., Chevrollier, A., Ferre, M., Guillet, V., Gueguen, N., Loiseau, D., de Crescenzo, M.A., Verny, C., Procaccio, V., Lenaers, G., Reynier, P., 2009. OPA1-associated disorders: phenotypes and pathophysiology. *Int J Biochem Cell Biol*. 41, 1855-65.
- Andreyev, A.Y., Kushnareva, Y.E., Starkov, A.A., 2005. Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc)*. 70, 200-14.
- Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., Mann, D., Tsuchiya, K., Yoshida, M., Hashizume, Y., Oda, T., 2006. TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochem Biophys Res Commun*. 351, 602-11.
- Arai, T., Mackenzie, I.R., Hasegawa, M., Nonaka, T., Niizato, K., Tsuchiya, K., Iritani, S., Onaya, M., Akiyama, H., 2009. Phosphorylated TDP-43 in Alzheimer's disease and dementia with Lewy bodies. *Acta Neuropathol*. 117, 125-36.
- Ayala, Y.M., Pantano, S., D'Ambrogio, A., Buratti, E., Brindisi, A., Marchetti, C., Romano, M., Baralle, F.E., 2005. Human, Drosophila, and C.elegans TDP43: nucleic acid binding properties and splicing regulatory function. *J Mol Biol*. 348, 575-88.
- Ayala, Y.M., Misteli, T., Baralle, F.E., 2008. TDP-43 regulates retinoblastoma protein phosphorylation through the repression of cyclin-dependent kinase 6 expression. *Proc Natl Acad Sci U S A*. 105, 3785-9.
- Ayala, Y.M., Zago, P., D'Ambrogio, A., Xu, Y.F., Petrucelli, L., Buratti, E., Baralle, F.E., 2008. Structural determinants of the cellular localization and shuttling of TDP-43. *J Cell Sci*. 121, 3778-85.
- Baillet, A., Chantepredrix, V., Trocme, C., Casez, P., Garrel, C., Besson, G., 2010. The role of oxidative stress in amyotrophic lateral sclerosis and Parkinson's disease. *Neurochem Res*. 35, 1530-7.
- Ban, T., Heymann, J.A., Song, Z., Hinshaw, J.E., Chan, D.C., 2010. OPA1 disease alleles causing dominant optic atrophy have defects in cardiolipin-stimulated GTP hydrolysis and membrane tubulation. *Hum Mol Genet*. 19, 2113-22.
- Barber, S.C., Mead, R.J., Shaw, P.J., 2006. Oxidative stress in ALS: a mechanism of neurodegeneration and a therapeutic target. *Biochim Biophys Acta*. 1762, 1051-67.
- Barber, S.C., Shaw, P.J., 2010. Oxidative stress in ALS: key role in motor neuron injury and therapeutic target. *Free Radic Biol Med*. 48, 629-41.
- Barmada, S.J., Skibinski, G., Korb, E., Rao, E.J., Wu, J.Y., Finkbeiner, S., 2010. Cytoplasmic mislocalization of TDP-43 is toxic to neurons and enhanced

- by a mutation associated with familial amyotrophic lateral sclerosis. *J Neurosci.* 30, 639-49.
- Bartley, M.G., Marquardt, K., Kirchhof, D., Wilkins, H.M., Patterson, D., Linseman, D.A., 2012. Overexpression of amyloid-beta protein precursor induces mitochondrial oxidative stress and activates the intrinsic apoptotic cascade. *J Alzheimers Dis.* 28, 855-68.
- Basu, A., Lu, D., Sun, B., Moor, A.N., Akkaraju, G.R., Huang, J., 2002. Proteolytic activation of protein kinase C-epsilon by caspase-mediated processing and transduction of antiapoptotic signals. *J Biol Chem.* 277, 41850-6.
- Bermejo, P., Martin-Aragon, S., Benedi, J., Susin, C., Felici, E., Gil, P., Ribera, J.M., Villar, A.M., 2008. Peripheral levels of glutathione and protein oxidation as markers in the development of Alzheimer's disease from Mild Cognitive Impairment. *Free Radic Res.* 42, 162-70.
- Bleazard, W., McCaffery, J.M., King, E.J., Bale, S., Mozdy, A., Tieu, Q., Nunnari, J., Shaw, J.M., 1999. The dynamin-related GTPase Dnm1 regulates mitochondrial fission in yeast. *Nat Cell Biol.* 1, 298-304.
- Bossy-Wetzel, E., Barsoum, M.J., Godzik, A., Schwarzenbacher, R., Lipton, S.A., 2003. Mitochondrial fission in apoptosis, neurodegeneration and aging. *Curr Opin Cell Biol.* 15, 706-16.
- Brady, O.A., Meng, P., Zheng, Y., Mao, Y., Hu, F., 2011. Regulation of TDP-43 aggregation by phosphorylation and p62/SQSTM1. *J Neurochem.* 116, 248-59.
- Brandmeir, N.J., Geser, F., Kwong, L.K., Zimmerman, E., Qian, J., Lee, V.M., Trojanowski, J.Q., 2008. Severe subcortical TDP-43 pathology in sporadic frontotemporal lobar degeneration with motor neuron disease. *Acta Neuropathol.* 115, 123-31.
- Buratti, E., Dork, T., Zuccato, E., Pagani, F., Romano, M., Baralle, F.E., 2001. Nuclear factor TDP-43 and SR proteins promote in vitro and in vivo CFTR exon 9 skipping. *EMBO J.* 20, 1774-84.
- Buratti, E., Brindisi, A., Giombi, M., Tisminetzky, S., Ayala, Y.M., Baralle, F.E., 2005. TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: an important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing. *J Biol Chem.* 280, 37572-84.
- Buratti, E., Baralle, F.E., 2008. Multiple roles of TDP-43 in gene expression, splicing regulation, and human disease. *Front Biosci.* 13, 867-78.
- Buratti, E., De Conti, L., Stuani, C., Romano, M., Baralle, M., Baralle, F., 2010. Nuclear factor TDP-43 can affect selected microRNA levels. *FEBS J.* 277, 2268-81.
- Butterfield, D.A., Swomley, A.M., Sultana, R., 2013. Amyloid beta-peptide (1-42)-induced oxidative stress in Alzheimer disease: importance in disease pathogenesis and progression. *Antioxid Redox Signal.* 19, 823-35.

- Cairns, N.J., Neumann, M., Bigio, E.H., Holm, I.E., Troost, D., Hatanpaa, K.J., Foong, C., White, C.L., 3rd, Schneider, J.A., Kretzschmar, H.A., Carter, D., Taylor-Reinwald, L., Paulsmeyer, K., Strider, J., Gitcho, M., Goate, A.M., Morris, J.C., Mishra, M., Kwong, L.K., Stieber, A., Xu, Y., Forman, M.S., Trojanowski, J.Q., Lee, V.M., Mackenzie, I.R., 2007. TDP-43 in familial and sporadic frontotemporal lobar degeneration with ubiquitin inclusions. *Am J Pathol.* 171, 227-40.
- Campello, S., Scorrano, L., 2010. Mitochondrial shape changes: orchestrating cell pathophysiology. *EMBO Rep.* 11, 678-84.
- Cappelletti, G., Surrey, T., Maci, R., 2005. The parkinsonism producing neurotoxin MPP+ affects microtubule dynamics by acting as a destabilising factor. *FEBS Lett.* 579, 4781-6.
- Cardoso, S.M., Oliveira, C.R., 2003. Glutathione cycle impairment mediates A beta-induced cell toxicity. *Free Radic Res.* 37, 241-50.
- Chaban, Y., Boekema, E.J., Dudkina, N.V., 2014. Structures of mitochondrial oxidative phosphorylation supercomplexes and mechanisms for their stabilisation. *Biochim Biophys Acta.* 1837, 418-26.
- Chakravarti, B., Chakravarti, D.N., 2007. Oxidative modification of proteins: age-related changes. *Gerontology.* 53, 128-39.
- Chan, D.C., 2012. Fusion and fission: interlinked processes critical for mitochondrial health. *Annu Rev Genet.* 46, 265-87.
- Chang, C.R., Blackstone, C., 2010. Dynamic regulation of mitochondrial fission through modification of the dynamin-related protein Drp1. *Ann N Y Acad Sci.* 1201, 34-9.
- Chen, Z., Lash, L.H., 1998. Evidence for mitochondrial uptake of glutathione by dicarboxylate and 2-oxoglutarate carriers. *J Pharmacol Exp Ther.* 285, 608-18.
- Chen, Y.R., Meyer, C.F., Ahmed, B., Yao, Z., Tan, T.H., 1999. Caspase-mediated cleavage and functional changes of hematopoietic progenitor kinase 1 (HPK1). *Oncogene.* 18, 7370-7.
- Chen, H., Detmer, S.A., Ewald, A.J., Griffin, E.E., Fraser, S.E., Chan, D.C., 2003. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. *J Cell Biol.* 160, 189-200.
- Chen, H., Chomyn, A., Chan, D.C., 2005. Disruption of fusion results in mitochondrial heterogeneity and dysfunction. *J Biol Chem.* 280, 26185-92.
- Chen-Plotkin, A.S., Lee, V.M., Trojanowski, J.Q., 2010. TAR DNA-binding protein 43 in neurodegenerative disease. *Nat Rev Neurol.* 6, 211-20.
- Chevrollier, A., Guillet, V., Loiseau, D., Gueguen, N., de Crescenzo, M.A., Verny, C., Ferre, M., Dollfus, H., Odent, S., Milea, D., Goizet, C., Amati-Bonneau, P., Procaccio, V., Bonneau, D., Reynier, P., 2008. Hereditary optic neuropathies share a common mitochondrial coupling defect. *Ann Neurol.* 63, 794-8.
- Cho, D.H., Nakamura, T., Lipton, S.A., 2010. Mitochondrial dynamics in cell death and neurodegeneration. *Cell Mol Life Sci.* 67, 3435-47.

- Chung, K.K., Thomas, B., Li, X., Pletnikova, O., Troncoso, J.C., Marsh, L., Dawson, V.L., Dawson, T.M., 2004. S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science*. 304, 1328-31.
- Cipolat, S., Martins de Brito, O., Dal Zilio, B., Scorrano, L., 2004. OPA1 requires mitofusin 1 to promote mitochondrial fusion. *Proc Natl Acad Sci U S A*. 101, 15927-32.
- Cipolat, S., Rudka, T., Hartmann, D., Costa, V., Serneels, L., Craessaerts, K., Metzger, K., Frezza, C., Annaert, W., D'Adamio, L., Derks, C., Dejaegere, T., Pellegrini, L., D'Hooge, R., Scorrano, L., De Strooper, B., 2006. Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. *Cell*. 126, 163-75.
- Coll, O., Colell, A., Garcia-Ruiz, C., Kaplowitz, N., Fernandez-Checa, J.C., 2003. Sensitivity of the 2-oxoglutarate carrier to alcohol intake contributes to mitochondrial glutathione depletion. *Hepatology*. 38, 692-702.
- Cooke, M.S., Evans, M.D., Dizdaroglu, M., Lunec, J., 2003. Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J*. 17, 1195-214.
- Corral-Debrinski, M., Horton, T., Lott, M.T., Shoffner, J.M., Beal, M.F., Wallace, D.C., 1992. Mitochondrial DNA deletions in human brain: regional variability and increase with advanced age. *Nat Genet*. 2, 324-9.
- Cortopassi, G.A., Arnheim, N., 1990. Detection of a specific mitochondrial DNA deletion in tissues of older humans. *Nucleic Acids Res*. 18, 6927-33.
- Coskun, P.E., Beal, M.F., Wallace, D.C., 2004. Alzheimer's brains harbor somatic mtDNA control-region mutations that suppress mitochondrial transcription and replication. *Proc Natl Acad Sci U S A*. 101, 10726-31.
- Cribbs, J.T., Strack, S., 2007. Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. *EMBO Rep*. 8, 939-44.
- D'Alessandro, G., Calcagno, E., Tartari, S., Rizzardini, M., Invernizzi, R.W., Cantoni, L., 2011. Glutamate and glutathione interplay in a motor neuronal model of amyotrophic lateral sclerosis reveals altered energy metabolism. *Neurobiol Dis*. 43, 346-55.
- Darios, F., Corti, O., Lucking, C.B., Hampe, C., Muriel, M.P., Abbas, N., Gu, W.J., Hirsch, E.C., Rooney, T., Ruberg, M., Brice, A., 2003. Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death. *Hum Mol Genet*. 12, 517-26.
- Davidson, Y., Kelley, T., Mackenzie, I.R., Pickering-Brown, S., Du Plessis, D., Neary, D., Snowden, J.S., Mann, D.M., 2007. Ubiquitinated pathological lesions in frontotemporal lobar degeneration contain the TAR DNA-binding protein, TDP-43. *Acta Neuropathol*. 113, 521-33.
- Davies, V.J., Hollins, A.J., Piechota, M.J., Yip, W., Davies, J.R., White, K.E., Nicols, P.P., Boulton, M.E., Votruba, M., 2007. Opa1 deficiency in a mouse model of autosomal dominant optic atrophy impairs mitochondrial

- morphology, optic nerve structure and visual function. *Hum Mol Genet.* 16, 1307-18.
- Davis, R.J., 2000. Signal transduction by the JNK group of MAP kinases. *Cell.* 103, 239-52.
- Day, C.L., Puthalakath, H., Skea, G., Strasser, A., Barsukov, I., Lian, L.Y., Huang, D.C., Hinds, M.G., 2004. Localization of dynein light chains 1 and 2 and their pro-apoptotic ligands. *Biochem J.* 377, 597-605.
- Del Bo, R., Bordonni, A., Martinelli Boneschi, F., Crimi, M., Sciacco, M., Bresolin, N., Scarlato, G., Comi, G.P., 2002. Evidence and age-related distribution of mtDNA D-loop point mutations in skeletal muscle from healthy subjects and mitochondrial patients. *J Neurol Sci.* 202, 85-91.
- Delettre, C., Lenaers, G., Griffoin, J.M., Gigarel, N., Lorenzo, C., Belenguer, P., Pelloquin, L., Grosgeorge, J., Turc-Carel, C., Perret, E., Astarie-Dequeker, C., Lasquelléc, L., Arnaud, B., Ducommun, B., Kaplan, J., Hamel, C.P., 2000. Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. *Nat Genet.* 26, 207-10.
- Delettre, C., Griffoin, J.M., Kaplan, J., Dollfus, H., Lorenz, B., Faivre, L., Lenaers, G., Belenguer, P., Hamel, C.P., 2001. Mutation spectrum and splicing variants in the OPA1 gene. *Hum Genet.* 109, 584-91.
- Deng, H., Dodson, M.W., Huang, H., Guo, M., 2008. The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in *Drosophila*. *Proc Natl Acad Sci U S A.* 105, 14503-8.
- Detmer, S.A., Chan, D.C., 2007. Functions and dysfunctions of mitochondrial dynamics. *Nat Rev Mol Cell Biol.* 8, 870-9.
- DeVay, R.M., Dominguez-Ramirez, L., Lackner, L.L., Hoppins, S., Stahlberg, H., Nunnari, J., 2009. Coassembly of Mgm1 isoforms requires cardiolipin and mediates mitochondrial inner membrane fusion. *J Cell Biol.* 186, 793-803.
- Dickson, D.W., Josephs, K.A., Amador-Ortiz, C., 2007. TDP-43 in differential diagnosis of motor neuron disorders. *Acta Neuropathol.* 114, 71-9.
- DiMauro, S., Andreu, A.L., 2000. Mutations in mtDNA: are we scraping the bottom of the barrel? *Brain Pathol.* 10, 431-41.
- Dormann, D., Capell, A., Carlson, A.M., Shankaran, S.S., Rodde, R., Neumann, M., Kremmer, E., Matsuwaki, T., Yamanouchi, K., Nishihara, M., Haass, C., 2009. Proteolytic processing of TAR DNA binding protein-43 by caspases produces C-terminal fragments with disease defining properties independent of progranulin. *J Neurochem.* 110, 1082-94.
- Duvezin-Caubet, S., Jagasia, R., Wagener, J., Hofmann, S., Trifunovic, A., Hansson, A., Chomyn, A., Bauer, M.F., Attardi, G., Larsson, N.G., Neupert, W., Reichert, A.S., 2006. Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. *J Biol Chem.* 281, 37972-9.
- Duvezin-Caubet, S., Koppen, M., Wagener, J., Zick, M., Israel, L., Bernacchia, A., Jagasia, R., Rugarli, E.I., Imhof, A., Neupert, W., Langer, T., Reichert, A.S., 2007. OPA1 processing reconstituted in yeast depends on the

- subunit composition of the m-AAA protease in mitochondria. *Mol Biol Cell*. 18, 3582-90.
- Ehstes, S., Raschke, I., Mancuso, G., Bernacchia, A., Geimer, S., Tondera, D., Martinou, J.C., Westermann, B., Rugarli, E.I., Langer, T., 2009. Regulation of OPA1 processing and mitochondrial fusion by m-AAA protease isoenzymes and OMA1. *J Cell Biol*. 187, 1023-36.
- Esmaeili, M.A., Panahi, M., Yadav, S., Hennings, L., Kiaei, M., 2013. Premature death of TDP-43 (A315T) transgenic mice due to gastrointestinal complications prior to development of full neurological symptoms of amyotrophic lateral sclerosis. *Int J Exp Pathol*. 94, 56-64.
- Evans, M.D., Dizdaroglu, M., Cooke, M.S., 2004. Oxidative DNA damage and disease: induction, repair and significance. *Mutat Res*. 567, 1-61.
- Ferrante, R.J., Browne, S.E., Shinobu, L.A., Bowling, A.C., Baik, M.J., MacGarvey, U., Kowall, N.W., Brown, R.H., Jr., Beal, M.F., 1997. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *J Neurochem*. 69, 2064-74.
- Filipcik, P., Cente, M., Ferencik, M., Hulin, I., Novak, M., 2006. The role of oxidative stress in the pathogenesis of Alzheimer's disease. *Bratisl Lek Listy*. 107, 384-94.
- Fitzmaurice, P.S., Shaw, I.C., Kleiner, H.E., Miller, R.T., Monks, T.J., Lau, S.S., Mitchell, J.D., Lynch, P.G., 1996. Evidence for DNA damage in amyotrophic lateral sclerosis. *Muscle Nerve*. 19, 797-8.
- Frezza, C., Cipolat, S., Martins de Brito, O., Micaroni, M., Beznoussenko, G.V., Rudka, T., Bartoli, D., Polishuck, R.S., Danial, N.N., De Strooper, B., Scorrano, L., 2006. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell*. 126, 177-89.
- Gandre-Babbe, S., van der Bliek, A.M., 2008. The novel tail-anchored membrane protein Mff controls mitochondrial and peroxisomal fission in mammalian cells. *Mol Biol Cell*. 19, 2402-12.
- Gendron, T.F., Petrucelli, L., 2011. Rodent models of TDP-43 proteinopathy: investigating the mechanisms of TDP-43-mediated neurodegeneration. *J Mol Neurosci*. 45, 486-99.
- Geser, F., Brandmeir, N.J., Kwong, L.K., Martinez-Lage, M., Elman, L., McCluskey, L., Xie, S.X., Lee, V.M., Trojanowski, J.Q., 2008. Evidence of multisystem disorder in whole-brain map of pathological TDP-43 in amyotrophic lateral sclerosis. *Arch Neurol*. 65, 636-41.
- Ghosh, D., LeVault, K.R., Barnett, A.J., Brewer, G.J., 2012. A reversible early oxidized redox state that precedes macromolecular ROS damage in aging nontransgenic and 3xTg-AD mouse neurons. *J Neurosci*. 32, 5821-32.
- Ghosh, D., LeVault, K.R., Brewer, G.J., 2014. Dual-energy precursor and nuclear erythroid-related factor 2 activator treatment additively improve redox glutathione levels and neuron survival in aging and Alzheimer mouse neurons upstream of reactive oxygen species. *Neurobiol Aging*. 35, 179-90.

- Giordana, M.T., Piccinini, M., Grifoni, S., De Marco, G., Vercellino, M., Magistrello, M., Pellerino, A., Buccinna, B., Lupino, E., Rinaudo, M.T., 2010. TDP-43 redistribution is an early event in sporadic amyotrophic lateral sclerosis. *Brain Pathol.* 20, 351-60.
- Gray, J.J., Zommer, A.E., Bouchard, R.J., Duval, N., Blackstone, C., Linseman, D.A., 2013. N-terminal cleavage of the mitochondrial fusion GTPase OPA1 occurs via a caspase-independent mechanism in cerebellar granule neurons exposed to oxidative or nitrosative stress. *Brain Res.* 1494, 28-43.
- Griffin, E.E., Graumann, J., Chan, D.C., 2005. The WD40 protein Caf4p is a component of the mitochondrial fission machinery and recruits Dnm1p to mitochondria. *J Cell Biol.* 170, 237-48.
- Griffith, O.W., Meister, A., 1985. Origin and turnover of mitochondrial glutathione. *Proc Natl Acad Sci U S A.* 82, 4668-72.
- Griparic, L., van der Wel, N.N., Orozco, I.J., Peters, P.J., van der Bliek, A.M., 2004. Loss of the intermembrane space protein Mgm1/OPA1 induces swelling and localized constrictions along the lengths of mitochondria. *J Biol Chem.* 279, 18792-8.
- Griparic, L., van der Bliek, A.M., 2005. Assay and properties of the mitochondrial dynamin related protein Opa1. *Methods Enzymol.* 404, 620-31.
- Griparic, L., Kanazawa, T., van der Bliek, A.M., 2007. Regulation of the mitochondrial dynamin-like protein Opa1 by proteolytic cleavage. *J Cell Biol.* 178, 757-64.
- Guillery, O., Malka, F., Landes, T., Guillou, E., Blackstone, C., Lombes, A., Belenguer, P., Arnoult, D., Rojo, M., 2008. Metalloprotease-mediated OPA1 processing is modulated by the mitochondrial membrane potential. *Biol Cell.* 100, 315-25.
- Guillou, E., Bousquet, C., Daloyau, M., Emorine, L.J., Belenguer, P., 2005. Msp1p is an intermembrane space dynamin-related protein that mediates mitochondrial fusion in a Dnm1p-dependent manner in *S. pombe*. *FEBS Lett.* 579, 1109-16.
- Handy, D.E., Loscalzo, J., 2012. Redox regulation of mitochondrial function. *Antioxid Redox Signal.* 16, 1323-67.
- Harbison, R.A., Ryan, K.R., Wilkins, H.M., Schroeder, E.K., Loucks, F.A., Bouchard, R.J., Linseman, D.A., 2011. Calpain plays a central role in 1-methyl-4-phenylpyridinium (MPP+)-induced neurotoxicity in cerebellar granule neurons. *Neurotox Res.* 19, 374-88.
- Hasegawa, M., Arai, T., Akiyama, H., Nonaka, T., Mori, H., Hashimoto, T., Yamazaki, M., Oyanagi, K., 2007. TDP-43 is deposited in the Guam parkinsonism-dementia complex brains. *Brain.* 130, 1386-94.
- Hasegawa, M., Arai, T., Nonaka, T., Kametani, F., Yoshida, M., Hashizume, Y., Beach, T.G., Buratti, E., Baralle, F., Morita, M., Nakano, I., Oda, T., Tsuchiya, K., Akiyama, H., 2008. Phosphorylated TDP-43 in

- frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Ann Neurol.* 64, 60-70.
- He, Y., Smith, R., 2009. Nuclear functions of heterogeneous nuclear ribonucleoproteins A/B. *Cell Mol Life Sci.* 66, 1239-56.
- Hensley, K., Carney, J.M., Mattson, M.P., Aksenova, M., Harris, M., Wu, J.F., Floyd, R.A., Butterfield, D.A., 1994. A model for beta-amyloid aggregation and neurotoxicity based on free radical generation by the peptide: relevance to Alzheimer disease. *Proc Natl Acad Sci U S A.* 91, 3270-4.
- Herdewyn, S., Cirillo, C., Van Den Bosch, L., Robberecht, W., Vanden Berghe, P., Van Damme, P., 2014. Prevention of intestinal obstruction reveals progressive neurodegeneration in mutant TDP-43 (A315T) mice. *Mol Neurodegener.* 9, 24.
- Herlan, M., Vogel, F., Bornhovd, C., Neupert, W., Reichert, A.S., 2003. Processing of Mgm1 by the rhomboid-type protease Pcp1 is required for maintenance of mitochondrial morphology and of mitochondrial DNA. *J Biol Chem.* 278, 27781-8.
- Higashi, S., Iseki, E., Yamamoto, R., Minegishi, M., Hino, H., Fujisawa, K., Togo, T., Katsuse, O., Uchikado, H., Furukawa, Y., Kosaka, K., Arai, H., 2007. Concurrence of TDP-43, tau and alpha-synuclein pathology in brains of Alzheimer's disease and dementia with Lewy bodies. *Brain Res.* 1184, 284-94.
- Hodges, J.R., Davies, R.R., Xuereb, J.H., Casey, B., Broe, M., Bak, T.H., Kril, J.J., Halliday, G.M., 2004. Clinicopathological correlates in frontotemporal dementia. *Ann Neurol.* 56, 399-406.
- Hong, K., Li, Y., Duan, W., Guo, Y., Jiang, H., Li, W., Li, C., 2012. Full-length TDP-43 and its C-terminal fragments activate mitophagy in NSC34 cell line. *Neurosci Lett.* 530, 144-9.
- Huang, C.Y., Wu, Y.M., Hsu, C.Y., Lee, W.S., Lai, M.D., Lu, T.J., Huang, C.L., Leu, T.H., Shih, H.M., Fang, H.I., Robinson, D.R., Kung, H.J., Yuan, C.J., 2002. Caspase activation of mammalian sterile 20-like kinase 3 (Mst3). Nuclear translocation and induction of apoptosis. *J Biol Chem.* 277, 34367-74.
- Hughey, C.C., Hittel, D.S., Johnsen, V.L., Shearer, J., 2011. Respiriometric oxidative phosphorylation assessment in saponin-permeabilized cardiac fibers. *J Vis Exp.*
- Igaz, L.M., Kwong, L.K., Xu, Y., Truax, A.C., Uryu, K., Neumann, M., Clark, C.M., Elman, L.B., Miller, B.L., Grossman, M., McCluskey, L.F., Trojanowski, J.Q., Lee, V.M., 2008. Enrichment of C-terminal fragments in TAR DNA-binding protein-43 cytoplasmic inclusions in brain but not in spinal cord of frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Am J Pathol.* 173, 182-94.
- Igaz, L.M., Kwong, L.K., Chen-Plotkin, A., Winton, M.J., Unger, T.L., Xu, Y., Neumann, M., Trojanowski, J.Q., Lee, V.M., 2009. Expression of TDP-43

- C-terminal Fragments in Vitro Recapitulates Pathological Features of TDP-43 Proteinopathies. *J Biol Chem.* 284, 8516-24.
- Ishihara, N., Fujita, Y., Oka, T., Mihara, K., 2006. Regulation of mitochondrial morphology through proteolytic cleavage of OPA1. *EMBO J.* 25, 2966-77.
- Jaarsma, D., Rognoni, F., van Duijn, W., Verspaget, H.W., Haasdijk, E.D., Holstege, J.C., 2001. CuZn superoxide dismutase (SOD1) accumulates in vacuolated mitochondria in transgenic mice expressing amyotrophic lateral sclerosis-linked SOD1 mutations. *Acta Neuropathol.* 102, 293-305.
- Jenner, P., 2003. Oxidative stress in Parkinson's disease. *Ann Neurol.* 53 Suppl 3, S26-36; discussion S36-8.
- Joseph, A.M., Adhiketty, P.J., Buford, T.W., Wohlgemuth, S.E., Lees, H.A., Nguyen, L.M., Aranda, J.M., Sandesara, B.D., Pahor, M., Manini, T.M., Marzetti, E., Leeuwenburgh, C., 2012. The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. *Aging Cell.* 11, 801-9.
- Kamga, C.K., Zhang, S.X., Wang, Y., 2010. Dicarboxylate carrier-mediated glutathione transport is essential for reactive oxygen species homeostasis and normal respiration in rat brain mitochondria. *Am J Physiol Cell Physiol.* 299, C497-505.
- Kane, D.A., Lin, C.T., Anderson, E.J., Kwak, H.B., Cox, J.H., Brophy, P.M., Hickner, R.C., Neufer, P.D., Cortright, R.N., 2011. Progesterone increases skeletal muscle mitochondrial H₂O₂ emission in nonmenopausal women. *Am J Physiol Endocrinol Metab.* 300, E528-35.
- Karbowski, M., Lee, Y.J., Gaume, B., Jeong, S.Y., Frank, S., Nechushtan, A., Santel, A., Fuller, M., Smith, C.L., Youle, R.J., 2002. Spatial and temporal association of Bax with mitochondrial fission sites, Drp1, and Mfn2 during apoptosis. *J Cell Biol.* 159, 931-8.
- Kelsey, N., Hulick, W., Winter, A., Ross, E., Linseman, D., 2011. Neuroprotective effects of anthocyanins on apoptosis induced by mitochondrial oxidative stress. *Nutr Neurosci.* 14, 249-59.
- Knott, A.B., Perkins, G., Schwarzenbacher, R., Bossy-Wetzel, E., 2008. Mitochondrial fragmentation in neurodegeneration. *Nat Rev Neurosci.* 9, 505-18.
- Koch, A., Yoon, Y., Bonekamp, N.A., McNiven, M.A., Schrader, M., 2005. A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. *Mol Biol Cell.* 16, 5077-86.
- Kong, J., Xu, Z., 1998. Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1. *J Neurosci.* 18, 3241-50.
- Koshiba, T., Detmer, S.A., Kaiser, J.T., Chen, H., McCaffery, J.M., Chan, D.C., 2004. Structural basis of mitochondrial tethering by mitofusin complexes. *Science.* 305, 858-62.

- Krishnan, K.J., Reeve, A.K., Samuels, D.C., Chinnery, P.F., Blackwood, J.K., Taylor, R.W., Wanrooij, S., Spelbrink, J.N., Lightowlers, R.N., Turnbull, D.M., 2008. What causes mitochondrial DNA deletions in human cells? *Nat Genet.* 40, 275-9.
- Labrousse, A.M., Zappaterra, M.D., Rube, D.A., van der Blik, A.M., 1999. C. elegans dynamin-related protein DRP-1 controls severing of the mitochondrial outer membrane. *Mol Cell.* 4, 815-26.
- Landes, T., Emorine, L.J., Courilleau, D., Rojo, M., Belenguer, P., Arnaune-Pelloquin, L., 2010. The BH3-only Bnip3 binds to the dynamin Opa1 to promote mitochondrial fragmentation and apoptosis by distinct mechanisms. *EMBO Rep.* 11, 459-65.
- Landes, T., Leroy, I., Bertholet, A., Diot, A., Khosrobakhsh, F., Daloyau, M., Davezac, N., Miquel, M.C., Courilleau, D., Guillou, E., Olichon, A., Lenaers, G., Arnaune-Pelloquin, L., Emorine, L.J., Belenguer, P., 2010. OPA1 (dys)functions. *Semin Cell Dev Biol.* 21, 593-8.
- Landino, L.M., Hasan, R., McGaw, A., Cooley, S., Smith, A.W., Masselam, K., Kim, G., 2002. Peroxynitrite oxidation of tubulin sulfhydryls inhibits microtubule polymerization. *Arch Biochem Biophys.* 398, 213-20.
- Landino, L.M., Skreslet, T.E., Alston, J.A., 2004. Cysteine oxidation of tau and microtubule-associated protein-2 by peroxynitrite: modulation of microtubule assembly kinetics by the thioredoxin reductase system. *J Biol Chem.* 279, 35101-5.
- Lash, L.H., 2006. Mitochondrial glutathione transport: physiological, pathological and toxicological implications. *Chem Biol Interact.* 163, 54-67.
- Lebovitz, R.M., Zhang, H., Vogel, H., Cartwright, J., Jr., Dionne, L., Lu, N., Huang, S., Matzuk, M.M., 1996. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc Natl Acad Sci U S A.* 93, 9782-7.
- Lee, N., MacDonald, H., Reinhard, C., Halenbeck, R., Roulston, A., Shi, T., Williams, L.T., 1997. Activation of hPAK65 by caspase cleavage induces some of the morphological and biochemical changes of apoptosis. *Proc Natl Acad Sci U S A.* 94, 13642-7.
- Lee, Y.J., Jeong, S.Y., Karbowski, M., Smith, C.L., Youle, R.J., 2004. Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. *Mol Biol Cell.* 15, 5001-11.
- Lee, E.B., Lee, V.M., Trojanowski, J.Q., 2012. Gains or losses: molecular mechanisms of TDP43-mediated neurodegeneration. *Nat Rev Neurosci.* 13, 38-50.
- Li, Y., Huang, T.T., Carlson, E.J., Melov, S., Ursell, P.C., Olson, J.L., Noble, L.J., Yoshimura, M.P., Berger, C., Chan, P.H., Wallace, D.C., Epstein, C.J., 1995. Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet.* 11, 376-81.

- Li, H., Zhu, H., Xu, C.J., Yuan, J., 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*. 94, 491-501.
- Li, Y., Ray, P., Rao, E.J., Shi, C., Guo, W., Chen, X., Woodruff, E.A., 3rd, Fushimi, K., Wu, J.Y., 2010. A *Drosophila* model for TDP-43 proteinopathy. *Proc Natl Acad Sci U S A*. 107, 3169-74.
- Liachko, N.F., Guthrie, C.R., Kraemer, B.C., 2010. Phosphorylation promotes neurotoxicity in a *Caenorhabditis elegans* model of TDP-43 proteinopathy. *J Neurosci*. 30, 16208-19.
- Lin, M.T., Beal, M.F., 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*. 443, 787-95.
- Liu, J., Lillo, C., Jonsson, P.A., Vande Velde, C., Ward, C.M., Miller, T.M., Subramaniam, J.R., Rothstein, J.D., Marklund, S., Andersen, P.M., Brannstrom, T., Gredal, O., Wong, P.C., Williams, D.S., Cleveland, D.W., 2004. Toxicity of familial ALS-linked SOD1 mutants from selective recruitment to spinal mitochondria. *Neuron*. 43, 5-17.
- Lodi, R., Tonon, C., Valentino, M.L., Manners, D., Testa, C., Malucelli, E., La Morgia, C., Barboni, P., Carbonelli, M., Schimpf, S., Wissinger, B., Zeviani, M., Baruzzi, A., Liguori, R., Barbiroli, B., Carelli, V., 2011. Defective mitochondrial adenosine triphosphate production in skeletal muscle from patients with dominant optic atrophy due to OPA1 mutations. *Arch Neurol*. 68, 67-73.
- Lomen-Hoerth, C., Murphy, J., Langmore, S., Kramer, J.H., Olney, R.K., Miller, B., 2003. Are amyotrophic lateral sclerosis patients cognitively normal? *Neurology*. 60, 1094-7.
- Loucks, F.A., Schroeder, E.K., Zommer, A.E., Hilger, S., Kelsey, N.A., Bouchard, R.J., Blackstone, C., Brewster, J.L., Linseman, D.A., 2009. Caspases indirectly regulate cleavage of the mitochondrial fusion GTPase OPA1 in neurons undergoing apoptosis. *Brain Res*. 1250, 63-74.
- Lovell, M.A., Xie, C., Markesbery, W.R., 1998. Decreased glutathione transferase activity in brain and ventricular fluid in Alzheimer's disease. *Neurology*. 51, 1562-6.
- Lovell, M.A., Xiong, S., Xie, C., Davies, P., Markesbery, W.R., 2004. Induction of hyperphosphorylated tau in primary rat cortical neuron cultures mediated by oxidative stress and glycogen synthase kinase-3. *J Alzheimers Dis*. 6, 659-71; discussion 673-81.
- Lu, S.C., 1999. Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB J*. 13, 1169-83.
- Lu, J., Duan, W., Guo, Y., Jiang, H., Li, Z., Huang, J., Hong, K., Li, C., 2012. Mitochondrial dysfunction in human TDP-43 transfected NSC34 cell lines and the protective effect of dimethoxy curcumin. *Brain Res Bull*. 89, 185-90.
- Mackenzie, I.R., Bigio, E.H., Ince, P.G., Geser, F., Neumann, M., Cairns, N.J., Kwong, L.K., Forman, M.S., Ravits, J., Stewart, H., Eisen, A., McClusky,

- L., Kretzschmar, H.A., Monoranu, C.M., Highley, J.R., Kirby, J., Siddique, T., Shaw, P.J., Lee, V.M., Trojanowski, J.Q., 2007. Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. *Ann Neurol.* 61, 427-34.
- Markesbery, W.R., 1997. Oxidative stress hypothesis in Alzheimer's disease. *Free Radic Biol Med.* 23, 134-47.
- Marshall, L.E., Himes, R.H., 1978. Rotenone inhibition of tubulin self-assembly. *Biochim Biophys Acta.* 543, 590-4.
- Mattiazzi, M., D'Aurelio, M., Gajewski, C.D., Martushova, K., Kiaei, M., Beal, M.F., Manfredi, G., 2002. Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *J Biol Chem.* 277, 29626-33.
- McKernan, T.B., Woods, E.B., Lash, L.H., 1991. Uptake of glutathione by renal cortical mitochondria. *Arch Biochem Biophys.* 288, 653-63.
- Meeusen, S., McCaffery, J.M., Nunnari, J., 2004. Mitochondrial fusion intermediates revealed in vitro. *Science.* 305, 1747-52.
- Meeusen, S., DeVay, R., Block, J., Cassidy-Stone, A., Wayson, S., McCaffery, J.M., Nunnari, J., 2006. Mitochondrial inner-membrane fusion and crista maintenance requires the dynamin-related GTPase Mgm1. *Cell.* 127, 383-95.
- Meglei, G., McQuibban, G.A., 2009. The dynamin-related protein Mgm1p assembles into oligomers and hydrolyzes GTP to function in mitochondrial membrane fusion. *Biochemistry.* 48, 1774-84.
- Meister, A., 1995. Mitochondrial changes associated with glutathione deficiency. *Biochim Biophys Acta.* 1271, 35-42.
- Mercado, P.A., Ayala, Y.M., Romano, M., Buratti, E., Baralle, F.E., 2005. Depletion of TDP 43 overrides the need for exonic and intronic splicing enhancers in the human apoA-II gene. *Nucleic Acids Res.* 33, 6000-10.
- Merkwirth, C., Langer, T., 2009. Prohibitin function within mitochondria: essential roles for cell proliferation and cristae morphogenesis. *Biochim Biophys Acta.* 1793, 27-32.
- Meyerowitz, J., Parker, S.J., Vella, L.J., Ng, D., Price, K.A., Liddell, J.R., Caragounis, A., Li, Q.X., Masters, C.L., Nonaka, T., Hasegawa, M., Bogoyevitch, M.A., Kanninen, K.M., Crouch, P.J., White, A.R., 2011. C-Jun N-terminal kinase controls TDP-43 accumulation in stress granules induced by oxidative stress. *Mol Neurodegener.* 6, 57.
- Michikawa, Y., Mazzucchelli, F., Bresolin, N., Scarlato, G., Attardi, G., 1999. Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. *Science.* 286, 774-9.
- Misaka, T., Miyashita, T., Kubo, Y., 2002. Primary structure of a dynamin-related mouse mitochondrial GTPase and its distribution in brain, subcellular localization, and effect on mitochondrial morphology. *J Biol Chem.* 277, 15834-42.

- Mitra, K., Wunder, C., Roysam, B., Lin, G., Lippincott-Schwartz, J., 2009. A hyperfused mitochondrial state achieved at G1-S regulates cyclin E buildup and entry into S phase. *Proc Natl Acad Sci U S A*. 106, 11960-5.
- Moisse, K., Mephram, J., Volkening, K., Welch, I., Hill, T., Strong, M.J., 2009. Cytosolic TDP-43 expression following axotomy is associated with caspase 3 activation in NFL-/- mice: support for a role for TDP-43 in the physiological response to neuronal injury. *Brain Res*. 1296, 176-86.
- Mori, F., Tanji, K., Zhang, H.X., Nishihira, Y., Tan, C.F., Takahashi, H., Wakabayashi, K., 2008. Maturation process of TDP-43-positive neuronal cytoplasmic inclusions in amyotrophic lateral sclerosis with and without dementia. *Acta Neuropathol*. 116, 193-203.
- Mozdy, A.D., McCaffery, J.M., Shaw, J.M., 2000. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. *J Cell Biol*. 151, 367-80.
- Nakashima-Yasuda, H., Uryu, K., Robinson, J., Xie, S.X., Hurtig, H., Duda, J.E., Arnold, S.E., Siderowf, A., Grossman, M., Leverenz, J.B., Woltjer, R., Lopez, O.L., Hamilton, R., Tsuang, D.W., Galasko, D., Masliah, E., Kaye, J., Clark, C.M., Montine, T.J., Lee, V.M., Trojanowski, J.Q., 2007. Co-morbidity of TDP-43 proteinopathy in Lewy body related diseases. *Acta Neuropathol*. 114, 221-9.
- Neumann, M., Sampathu, D.M., Kwong, L.K., Truax, A.C., Micsenyi, M.C., Chou, T.T., Bruce, J., Schuck, T., Grossman, M., Clark, C.M., McCluskey, L.F., Miller, B.L., Masliah, E., Mackenzie, I.R., Feldman, H., Feiden, W., Kretzschmar, H.A., Trojanowski, J.Q., Lee, V.M., 2006. Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science*. 314, 130-3.
- Neumann, M., Kwong, L.K., Lee, E.B., Kremmer, E., Flatley, A., Xu, Y., Forman, M.S., Troost, D., Kretzschmar, H.A., Trojanowski, J.Q., Lee, V.M., 2009. Phosphorylation of S409/410 of TDP-43 is a consistent feature in all sporadic and familial forms of TDP-43 proteinopathies. *Acta Neuropathol*. 117, 137-49.
- Newhouse, K., Hsuan, S.L., Chang, S.H., Cai, B., Wang, Y., Xia, Z., 2004. Rotenone-induced apoptosis is mediated by p38 and JNK MAP kinases in human dopaminergic SH-SY5Y cells. *Toxicol Sci*. 79, 137-46.
- Nguyen, D., Alavi, M.V., Kim, K.Y., Kang, T., Scott, R.T., Noh, Y.H., Lindsey, J.D., Wissinger, B., Ellisman, M.H., Weinreb, R.N., Perkins, G.A., Ju, W.K., 2011. A new vicious cycle involving glutamate excitotoxicity, oxidative stress and mitochondrial dynamics. *Cell Death Dis*. 2, e240.
- Nishimoto, Y., Ito, D., Yagi, T., Nihei, Y., Tsunoda, Y., Suzuki, N., 2010. Characterization of alternative isoforms and inclusion body of the TAR DNA-binding protein-43. *J Biol Chem*. 285, 608-19.
- Nunomura, A., Perry, G., Aliev, G., Hirai, K., Takeda, A., Balraj, E.K., Jones, P.K., Ghanbari, H., Wataya, T., Shimohama, S., Chiba, S., Atwood, C.S.,

- Petersen, R.B., Smith, M.A., 2001. Oxidative damage is the earliest event in Alzheimer disease. *J Neuropathol Exp Neurol.* 60, 759-67.
- Olichon, A., Emorine, L.J., Descoins, E., Pelloquin, L., Brichese, L., Gas, N., Guillou, E., Delettre, C., Valette, A., Hamel, C.P., Ducommun, B., Lenaers, G., Belenguer, P., 2002. The human dynamin-related protein OPA1 is anchored to the mitochondrial inner membrane facing the inter-membrane space. *FEBS Lett.* 523, 171-6.
- Olichon, A., Baricault, L., Gas, N., Guillou, E., Valette, A., Belenguer, P., Lenaers, G., 2003. Loss of OPA1 perturbs the mitochondrial inner membrane structure and integrity, leading to cytochrome c release and apoptosis. *J Biol Chem.* 278, 7743-6.
- Olichon, A., Elachouri, G., Baricault, L., Delettre, C., Belenguer, P., Lenaers, G., 2007. OPA1 alternate splicing uncouples an evolutionary conserved function in mitochondrial fusion from a vertebrate restricted function in apoptosis. *Cell Death Differ.* 14, 682-92.
- Oropesa, M., de la Mata, M., Maraver, J.G., Cordero, M.D., Cotan, D., Rodriguez-Hernandez, A., Dominguez-Monino, I., de Miguel, M., Navas, P., Sanchez-Alcazar, J.A., 2011. Apoptotic microtubule network organization and maintenance depend on high cellular ATP levels and energized mitochondria. *Apoptosis.* 16, 404-24.
- Otera, H., Wang, C., Cleland, M.M., Setoguchi, K., Yokota, S., Youle, R.J., Mihara, K., 2010. Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. *J Cell Biol.* 191, 1141-58.
- Ou, S.H., Wu, F., Harrich, D., Garcia-Martinez, L.F., Gaynor, R.B., 1995. Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs. *J Virol.* 69, 3584-96.
- Ow, Y.P., Green, D.R., Hao, Z., Mak, T.W., 2008. Cytochrome c: functions beyond respiration. *Nat Rev Mol Cell Biol.* 9, 532-42.
- Palacino, J.J., Sagi, D., Goldberg, M.S., Krauss, S., Motz, C., Wacker, M., Klose, J., Shen, J., 2004. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. *J Biol Chem.* 279, 18614-22.
- Palmer, C.S., Osellame, L.D., Laine, D., Koutsopoulos, O.S., Frazier, A.E., Ryan, M.T., 2011. MiD49 and MiD51, new components of the mitochondrial fission machinery. *EMBO Rep.* 12, 565-73.
- Park, Y.Y., Kim, S.B., Han, H.D., Sohn, B.H., Kim, J.H., Liang, J., Lu, Y., Rodriguez-Aguayo, C., Lopez-Berestein, G., Mills, G.B., Sood, A.K., Lee, J.S., 2013. Tat-activating regulatory DNA-binding protein regulates glycolysis in hepatocellular carcinoma by regulating the platelet isoform of phosphofructokinase through microRNA 520. *Hepatology.* 58, 182-91.
- Parker, S.J., Meyerowitz, J., James, J.L., Liddell, J.R., Crouch, P.J., Kanninen, K.M., White, A.R., 2012. Endogenous TDP-43 localized to stress granules can subsequently form protein aggregates. *Neurochem Int.* 60, 415-24.

- Pasinelli, P., Belford, M.E., Lennon, N., Bacsikai, B.J., Hyman, B.T., Trotti, D., Brown, R.H., Jr., 2004. Amyotrophic lateral sclerosis-associated SOD1 mutant proteins bind and aggregate with Bcl-2 in spinal cord mitochondria. *Neuron*. 43, 19-30.
- Perkins, G., Bossy-Wetzel, E., Ellisman, M.H., 2009. New insights into mitochondrial structure during cell death. *Exp Neurol*. 218, 183-92.
- Perry, G., Nunomura, A., Hirai, K., Zhu, X., Perez, M., Avila, J., Castellani, R.J., Atwood, C.S., Aliev, G., Sayre, L.M., Takeda, A., Smith, M.A., 2002. Is oxidative damage the fundamental pathogenic mechanism of Alzheimer's and other neurodegenerative diseases? *Free Radic Biol Med*. 33, 1475-9.
- Pesah, Y., Pham, T., Burgess, H., Middlebrooks, B., Verstreken, P., Zhou, Y., Harding, M., Bellen, H., Mardon, G., 2004. Drosophila parkin mutants have decreased mass and cell size and increased sensitivity to oxygen radical stress. *Development*. 131, 2183-94.
- Piko, L., Hougham, A.J., Bulpitt, K.J., 1988. Studies of sequence heterogeneity of mitochondrial DNA from rat and mouse tissues: evidence for an increased frequency of deletions/additions with aging. *Mech Ageing Dev*. 43, 279-93.
- Przedborski, S., Vila, M., 2003. The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model: a tool to explore the pathogenesis of Parkinson's disease. *Ann N Y Acad Sci*. 991, 189-98.
- Puthalakath, H., Huang, D.C., O'Reilly, L.A., King, S.M., Strasser, A., 1999. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. *Mol Cell*. 3, 287-96.
- Ramassamy, C., Averill, D., Beffert, U., Theroux, L., Lussier-Cacan, S., Cohn, J.S., Christen, Y., Schoofs, A., Davignon, J., Poirier, J., 2000. Oxidative insults are associated with apolipoprotein E genotype in Alzheimer's disease brain. *Neurobiol Dis*. 7, 23-37.
- Reddy, P.H., McWeeney, S., Park, B.S., Manczak, M., Gutala, R.V., Partovi, D., Jung, Y., Yau, V., Searles, R., Mori, M., Quinn, J., 2004. Gene expression profiles of transcripts in amyloid precursor protein transgenic mice: up-regulation of mitochondrial metabolism and apoptotic genes is an early cellular change in Alzheimer's disease. *Hum Mol Genet*. 13, 1225-40.
- Resende, R., Moreira, P.I., Proenca, T., Deshpande, A., Busciglio, J., Pereira, C., Oliveira, C.R., 2008. Brain oxidative stress in a triple-transgenic mouse model of Alzheimer disease. *Free Radic Biol Med*. 44, 2051-7.
- Riku, Y., Watanabe, H., Yoshida, M., Tatsumi, S., Mimuro, M., Iwasaki, Y., Katsuno, M., Iguchi, Y., Masuda, M., Senda, J., Ishigaki, S., Udagawa, T., Sobue, G., 2014. Lower motor neuron involvement in TAR DNA-binding protein of 43 kDa-related frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *JAMA Neurol*. 71, 172-9.
- Rizzardini, M., Lupi, M., Bernasconi, S., Mangolini, A., Cantoni, L., 2003. Mitochondrial dysfunction and death in motor neurons exposed to the glutathione-depleting agent ethacrynic acid. *J Neurol Sci*. 207, 51-8.

- Satoh, M., Hamamoto, T., Seo, N., Kagawa, Y., Endo, H., 2003. Differential sublocalization of the dynamin-related protein OPA1 isoforms in mitochondria. *Biochem Biophys Res Commun.* 300, 482-93.
- Saxton, W.M., Hollenbeck, P.J., 2012. The axonal transport of mitochondria. *J Cell Sci.* 125, 2095-104.
- Schapira, A.H., Cooper, J.M., Dexter, D., Clark, J.B., Jenner, P., Marsden, C.D., 1990. Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem.* 54, 823-7.
- Schwab, C., Arai, T., Hasegawa, M., Yu, S., McGeer, P.L., 2008. Colocalization of transactivation-responsive DNA-binding protein 43 and huntingtin in inclusions of Huntington disease. *J Neuropathol Exp Neurol.* 67, 1159-65.
- Sesaki, H., Jensen, R.E., 1999. Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. *J Cell Biol.* 147, 699-706.
- Sesaki, H., Southard, S.M., Yaffe, M.P., Jensen, R.E., 2003. Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane. *Mol Biol Cell.* 14, 2342-56.
- Shaw, P.J., Ince, P.G., Falkous, G., Mantle, D., 1995. Oxidative damage to protein in sporadic motor neuron disease spinal cord. *Ann Neurol.* 38, 691-5.
- Sherer, T.B., Richardson, J.R., Testa, C.M., Seo, B.B., Panov, A.V., Yagi, T., Matsuno-Yagi, A., Miller, G.W., Greenamyre, J.T., 2007. Mechanism of toxicity of pesticides acting at complex I: relevance to environmental etiologies of Parkinson's disease. *J Neurochem.* 100, 1469-79.
- Sheridan, C., Martin, S.J., 2010. Mitochondrial fission/fusion dynamics and apoptosis. *Mitochondrion.* 10, 640-8.
- Shidoji, Y., Hayashi, K., Komura, S., Ohishi, N., Yagi, K., 1999. Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. *Biochem Biophys Res Commun.* 264, 343-7.
- Smeyne, M., Smeyne, R.J., 2013. Glutathione metabolism and Parkinson's disease. *Free Radic Biol Med.* 62, 13-25.
- Smirnova, E., Griparic, L., Shurland, D.L., van der Bliek, A.M., 2001. Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell.* 12, 2245-56.
- Smith, M.A., Richey Harris, P.L., Sayre, L.M., Beckman, J.S., Perry, G., 1997. Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J Neurosci.* 17, 2653-7.
- Soderdahl, T., Enoksson, M., Lundberg, M., Holmgren, A., Ottersen, O.P., Orrenius, S., Bolcsfoldi, G., Cotgreave, I.A., 2003. Visualization of the compartmentalization of glutathione and protein-glutathione mixed disulfides in cultured cells. *FASEB J.* 17, 124-6.
- Song, D.D., Shults, C.W., Sisk, A., Rockenstein, E., Masliah, E., 2004. Enhanced substantia nigra mitochondrial pathology in human alpha-synuclein transgenic mice after treatment with MPTP. *Exp Neurol.* 186, 158-72.

- Song, Z., Chen, H., Fiket, M., Alexander, C., Chan, D.C., 2007. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. *J Cell Biol.* 178, 749-55.
- Song, Z., Ghochani, M., McCaffery, J.M., Frey, T.G., Chan, D.C., 2009. Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. *Mol Biol Cell.* 20, 3525-32.
- Song, W., Chen, J., Petrilli, A., Liot, G., Klinglmayr, E., Zhou, Y., Poquiz, P., Tjong, J., Pouladi, M.A., Hayden, M.R., Masliah, E., Ellisman, M., Rouiller, I., Schwarzenbacher, R., Bossy, B., Perkins, G., Bossy-Wetzel, E., 2011. Mutant huntingtin binds the mitochondrial fission GTPase dynamin-related protein-1 and increases its enzymatic activity. *Nat Med.* 17, 377-82.
- Spinazzi, M., Cazzola, S., Bortolozzi, M., Baracca, A., Loro, E., Casarin, A., Solaini, G., Sgarbi, G., Casalena, G., Cenacchi, G., Malena, A., Frezza, C., Carrara, F., Angelini, C., Scorrano, L., Salviati, L., Vergani, L., 2008. A novel deletion in the GTPase domain of OPA1 causes defects in mitochondrial morphology and distribution, but not in function. *Hum Mol Genet.* 17, 3291-302.
- Stojanovski, D., Koutsopoulos, O.S., Okamoto, K., Ryan, M.T., 2004. Levels of human Fis1 at the mitochondrial outer membrane regulate mitochondrial morphology. *J Cell Sci.* 117, 1201-10.
- Stribl, C., Samara, A., Trumbach, D., Peis, R., Neumann, M., Fuchs, H., Gailus-Durner, V., Hrabe de Angelis, M., Rathkolb, B., Wolf, E., Beckers, J., Horsch, M., Neff, F., Kremmer, E., Koob, S., Reichert, A.S., Hans, W., Rozman, J., Klingenspor, M., Aichler, M., Walch, A.K., Becker, L., Klopstock, T., Glasl, L., Holter, S.M., Wurst, W., Floss, T., 2014. Mitochondrial dysfunction and decrease in body weight of a transgenic knock-in mouse model for TDP-43. *J Biol Chem.* 289, 10769-84.
- Strong, M.J., Volkening, K., Hammond, R., Yang, W., Strong, W., Leysstra-Lantz, C., Shoesmith, C., 2007. TDP43 is a human low molecular weight neurofilament (hNFL) mRNA-binding protein. *Mol Cell Neurosci.* 35, 320-7.
- Suen, D.F., Norris, K.L., Youle, R.J., 2008. Mitochondrial dynamics and apoptosis. *Genes Dev.* 22, 1577-90.
- Takahashi, M., Mukai, H., Toshimori, M., Miyamoto, M., Ono, Y., 1998. Proteolytic activation of PKN by caspase-3 or related protease during apoptosis. *Proc Natl Acad Sci U S A.* 95, 11566-71.
- Takeuchi, H., Kobayashi, Y., Ishigaki, S., Doyu, M., Sobue, G., 2002. Mitochondrial localization of mutant superoxide dismutase 1 triggers caspase-dependent cell death in a cellular model of familial amyotrophic lateral sclerosis. *J Biol Chem.* 277, 50966-72.
- Tamagno, E., Parola, M., Bardini, P., Piccini, A., Borghi, R., Guglielmotto, M., Santoro, G., Davit, A., Danni, O., Smith, M.A., Perry, G., Tabaton, M., 2005. Beta-site APP cleaving enzyme up-regulation induced by 4-

- hydroxynonenal is mediated by stress-activated protein kinases pathways. *J Neurochem.* 92, 628-36.
- Tang, S., Le, P.K., Tse, S., Wallace, D.C., Huang, T., 2009. Heterozygous mutation of Opa1 in *Drosophila* shortens lifespan mediated through increased reactive oxygen species production. *PLoS One.* 4, e4492.
- Tashiro, Y., Urushitani, M., Inoue, H., Koike, M., Uchiyama, Y., Komatsu, M., Tanaka, K., Yamazaki, M., Abe, M., Misawa, H., Sakimura, K., Ito, H., Takahashi, R., 2012. Motor neuron-specific disruption of proteasomes, but not autophagy, replicates amyotrophic lateral sclerosis. *J Biol Chem.* 287, 42984-94.
- Tieu, Q., Nunnari, J., 2000. Mdv1p is a WD repeat protein that interacts with the dynamin-related GTPase, Dnm1p, to trigger mitochondrial division. *J Cell Biol.* 151, 353-66.
- Twig, G., Elorza, A., Molina, A.J., Mohamed, H., Wikstrom, J.D., Walzer, G., Stiles, L., Haigh, S.E., Katz, S., Las, G., Alroy, J., Wu, M., Py, B.F., Yuan, J., Deeney, J.T., Corkey, B.E., Shirihai, O.S., 2008. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. *EMBO J.* 27, 433-46.
- van Eersel, J., Ke, Y.D., Gladbach, A., Bi, M., Gotz, J., Kril, J.J., Ittner, L.M., 2011. Cytoplasmic accumulation and aggregation of TDP-43 upon proteasome inhibition in cultured neurons. *PLoS One.* 6, e22850.
- Varadarajan, S., Yatin, S., Aksenova, M., Butterfield, D.A., 2000. Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. *J Struct Biol.* 130, 184-208.
- Venkateshappa, C., Harish, G., Mahadevan, A., Srinivas Bharath, M.M., Shankar, S.K., 2012. Elevated oxidative stress and decreased antioxidant function in the human hippocampus and frontal cortex with increasing age: implications for neurodegeneration in Alzheimer's disease. *Neurochem Res.* 37, 1601-14.
- Voigt, A., Herholz, D., Fiesel, F.C., Kaur, K., Muller, D., Karsten, P., Weber, S.S., Kahle, P.J., Marquardt, T., Schulz, J.B., 2010. TDP-43-mediated neuron loss in vivo requires RNA-binding activity. *PLoS One.* 5, e12247.
- Wadey, A.L., Muyderman, H., Kwek, P.T., Sims, N.R., 2009. Mitochondrial glutathione uptake: characterization in isolated brain mitochondria and astrocytes in culture. *J Neurochem.* 109 Suppl 1, 101-8.
- Wallace, D.C., 2001. Mouse models for mitochondrial disease. *Am J Med Genet.* 106, 71-93.
- Wang, H.Y., Wang, I.F., Bose, J., Shen, C.K., 2004. Structural diversity and functional implications of the eukaryotic TDP gene family. *Genomics.* 83, 130-9.
- Wang, X., Su, B., Siedlak, S.L., Moreira, P.I., Fujioka, H., Wang, Y., Casadesus, G., Zhu, X., 2008. Amyloid-beta overproduction causes abnormal mitochondrial dynamics via differential modulation of mitochondrial fission/fusion proteins. *Proc Natl Acad Sci U S A.* 105, 19318-23.

- Wang, X., Su, B., Lee, H.G., Li, X., Perry, G., Smith, M.A., Zhu, X., 2009. Impaired balance of mitochondrial fission and fusion in Alzheimer's disease. *J Neurosci.* 29, 9090-103.
- Wang, W., Li, L., Lin, W.L., Dickson, D.W., Petrucelli, L., Zhang, T., Wang, X., 2013. The ALS disease-associated mutant TDP-43 impairs mitochondrial dynamics and function in motor neurons. *Hum Mol Genet.* 22, 4706-19.
- Wegorzewska, I., Bell, S., Cairns, N.J., Miller, T.M., Baloh, R.H., 2009. TDP-43 mutant transgenic mice develop features of ALS and frontotemporal lobar degeneration. *Proc Natl Acad Sci U S A.* 106, 18809-14.
- Whitworth, A.J., Theodore, D.A., Greene, J.C., Benes, H., Wes, P.D., Pallanck, L.J., 2005. Increased glutathione S-transferase activity rescues dopaminergic neuron loss in a *Drosophila* model of Parkinson's disease. *Proc Natl Acad Sci U S A.* 102, 8024-9.
- Wilkins, H.M., Marquardt, K., Lash, L.H., Linseman, D.A., 2012. Bcl-2 is a novel interacting partner for the 2-oxoglutarate carrier and a key regulator of mitochondrial glutathione. *Free Radic Biol Med.* 52, 410-9.
- Wilkins, H.M., Kirchhof, D., Manning, E., Joseph, J.W., Linseman, D.A., 2013. Mitochondrial glutathione transport is a key determinant of neuronal susceptibility to oxidative and nitrosative stress. *J Biol Chem.* 288, 5091-101.
- Wilkins, H.M., Brock, S., Gray, J.J., Linseman, D.A., 2014. Stable over-expression of the 2-oxoglutarate carrier enhances neuronal cell resistance to oxidative stress via Bcl-2-dependent mitochondrial GSH transport. *J Neurochem.* 130, 75-86.
- Winton, M.J., Igaz, L.M., Wong, M.M., Kwong, L.K., Trojanowski, J.Q., Lee, V.M., 2008. Disturbance of nuclear and cytoplasmic TAR DNA-binding protein (TDP-43) induces disease-like redistribution, sequestration, and aggregate formation. *J Biol Chem.* 283, 13302-9.
- Wong, E.D., Wagner, J.A., Gorsich, S.W., McCaffery, J.M., Shaw, J.M., Nunnari, J., 2000. The dynamin-related GTPase, Mgm1p, is an intermembrane space protein required for maintenance of fusion competent mitochondria. *J Cell Biol.* 151, 341-52.
- Xu, Y.F., Gendron, T.F., Zhang, Y.J., Lin, W.L., D'Alton, S., Sheng, H., Casey, M.C., Tong, J., Knight, J., Yu, X., Rademakers, R., Boylan, K., Hutton, M., McGowan, E., Dickson, D.W., Lewis, J., Petrucelli, L., 2010. Wild-type human TDP-43 expression causes TDP-43 phosphorylation, mitochondrial aggregation, motor deficits, and early mortality in transgenic mice. *J Neurosci.* 30, 10851-9.
- Yamaguchi, R., Lartigue, L., Perkins, G., Scott, R.T., Dixit, A., Kushnareva, Y., Kuwana, T., Ellisman, M.H., Newmeyer, D.D., 2008. Opa1-mediated cristae opening is Bax/Bak and BH3 dependent, required for apoptosis, and independent of Bak oligomerization. *Mol Cell.* 31, 557-69.

- Yamashita, M., Nonaka, T., Hirai, S., Miwa, A., Okado, H., Arai, T., Hosokawa, M., Akiyama, H., Hasegawa, M., 2014. Distinct pathways leading to TDP-43-induced cellular dysfunctions. *Hum Mol Genet.*
- Yang, C., Tan, W., Whittle, C., Qiu, L., Cao, L., Akbarian, S., Xu, Z., 2010. The C-terminal TDP-43 fragments have a high aggregation propensity and harm neurons by a dominant-negative mechanism. *PLoS One.* 5, e15878.
- Yoon, Y., Krueger, E.W., Oswald, B.J., McNiven, M.A., 2003. The mitochondrial protein hFis1 regulates mitochondrial fission in mammalian cells through an interaction with the dynamin-like protein DLP1. *Mol Cell Biol.* 23, 5409-20.
- Youle, R.J., van der Bliek, A.M., 2012. Mitochondrial fission, fusion, and stress. *Science.* 337, 1062-5.
- Yu, W., Sun, Y., Guo, S., Lu, B., 2011. The PINK1/Parkin pathway regulates mitochondrial dynamics and function in mammalian hippocampal and dopaminergic neurons. *Hum Mol Genet.* 20, 3227-40.
- Yuan, H., Gerencser, A.A., Liot, G., Lipton, S.A., Ellisman, M., Perkins, G.A., Bossy-Wetzel, E., 2007. Mitochondrial fission is an upstream and required event for bax foci formation in response to nitric oxide in cortical neurons. *Cell Death Differ.* 14, 462-71.
- Zanna, C., Ghelli, A., Porcelli, A.M., Karbowski, M., Youle, R.J., Schimpf, S., Wissinger, B., Pinti, M., Cossarizza, A., Vidoni, S., Valentino, M.L., Rugolo, M., Carelli, V., 2008. OPA1 mutations associated with dominant optic atrophy impair oxidative phosphorylation and mitochondrial fusion. *Brain.* 131, 352-67.
- Zhang, C., Baumer, A., Maxwell, R.J., Linnane, A.W., Nagley, P., 1992. Multiple mitochondrial DNA deletions in an elderly human individual. *FEBS Lett.* 297, 34-8.
- Zhang, Y.J., Xu, Y.F., Dickey, C.A., Buratti, E., Baralle, F., Bailey, R., Pickering-Brown, S., Dickson, D., Petrucelli, L., 2007. Progranulin mediates caspase-dependent cleavage of TAR DNA binding protein-43. *J Neurosci.* 27, 10530-4.
- Zhang, Y.J., Xu, Y.F., Cook, C., Gendron, T.F., Roettges, P., Link, C.D., Lin, W.L., Tong, J., Castanedes-Casey, M., Ash, P., Gass, J., Rangachari, V., Buratti, E., Baralle, F., Golde, T.E., Dickson, D.W., Petrucelli, L., 2009. Aberrant cleavage of TDP-43 enhances aggregation and cellular toxicity. *Proc Natl Acad Sci U S A.* 106, 7607-12.
- Zhang, Y.J., Gendron, T.F., Xu, Y.F., Ko, L.W., Yen, S.H., Petrucelli, L., 2010. Phosphorylation regulates proteasomal-mediated degradation and solubility of TAR DNA binding protein-43 C-terminal fragments. *Mol Neurodegener.* 5, 33.
- Zhu, P.P., Patterson, A., Lavoie, B., Stadler, J., Shoeb, M., Patel, R., Blackstone, C., 2003. Cellular localization, oligomerization, and membrane association of the hereditary spastic paraplegia 3A (SPG3A) protein atlastin. *J Biol Chem.* 278, 49063-71.

Appendix A: Abbreviations

AD, Alzheimer's disease
ADOA-1, autosomal dominant optic atrophy 1
ALS, Amyotrophic lateral sclerosis
APP, amyloid precursor protein
CC, coiled-coil
CCCP, carbonyl cyanide m-chlorophenyl hydrazone
CGNs, cerebellar granule neurons
CIC, citrate carrier/tricarboxylate carrier
CNS, central nervous system
DIC, dicarboxylate carrier
Dnm1, dynamin 1
DRP1, dynamin-related protein 1
EA, ethacrynic acid
ETC, electron transport chain
FIS1, fission 1
FTLD, Fronto temporal lobar degeneration
GED, GTPase effector domain
GPx, glutathione peroxidase
GR, glutathione reductase
GSH, glutathione
H₂O₂, hydrogen peroxide
HD, Huntington's disease
HR, heptad repeats
JNK, c-Jun N-terminal kinase
MAPK, mitogen activated protein kinase
MFF, mitochondrial fission factor
DRP1/2, Mitofusin 1/2
MiD49/51, mitochondrial dynamics protein
MIEF1, mitochondrial elongation factor 1
MIS, mitochondrial import signal
MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA, mitochondrial DNA
MTS, mitochondrial targeting sequence
NES, nuclear export signal
NLS, nuclear localization signal
OGC2, oxoglutarate carrier
OPA1, Optic atrophy 1
OXPHOS, oxidative phosphorylation
PARL, presenilin-associated rhomboid-like protease
PD, Parkinson's disease
PH, plextrin homology
RNS, reactive nitrogen species

ROS, reactive oxygen species
SOD, superoxide dismutase
TDP-43, TAR-DNA binding protein
TM, transmembrane domain
TPR, tetratricopeptide repeats

Appendix B- Publications

Chapter Two titled “: N-terminal cleavage of the mitochondrial fusion GTPase OPA1 occurs via a caspase-independent mechanism in cerebellar granule neurons exposed to oxidative or nitrosative stress” was published in *Brain Research*, 2013; 1494, 28-43.

Portions of Chapter Four titled “TDP-43 nuclear export is partially mediated by mitochondrial glutathione levels and c-Jun N –Terminal Kinase” were published in *Journal of Neurochemistry* 2014, 130, 75-86.